



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>A61K 49/00, 47/48, 51/08, 51/10</b>		A2	(11) International Publication Number: <b>WO 98/18495</b>																					
			(43) International Publication Date: <b>7 May 1998 (07.05.98)</b>																					
<p>(21) International Application Number: <b>PCT/GB97/02955</b></p> <p>(22) International Filing Date: <b>28 October 1997 (28.10.97)</b></p> <p>(30) Priority Data:</p> <table> <tr><td>9622365.6</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9622367.2</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9622366.4</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9700699.3</td><td>15 January 1997 (15.01.97)</td><td>GB</td></tr> <tr><td>9708265.5</td><td>24 April 1997 (24.04.97)</td><td>GB</td></tr> <tr><td>9711842.6</td><td>6 June 1997 (06.06.97)</td><td>GB</td></tr> <tr><td>9711845.9</td><td>6 June 1997 (06.06.97)</td><td>GB</td></tr> </table> <p>(71) Applicant (for GB only): <b>MARSDEN, John, Christopher [GB/GB]; Frank B. Dehn &amp; Co., 179 Queen Victoria Street, London EC4V 4EL (GB).</b></p> <p>(71) Applicant (for all designated States except US): <b>NYCOMED IMAGING AS [NO/NO]; Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO).</b></p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): <b>KLAVENESS, Jo [NO/NO]; Midtåsen 5, N-1166 Oslo (NO). RONGVED, Pål [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). HØGSET,</b></p>		9622365.6	28 October 1996 (28.10.96)	GB	9622367.2	28 October 1996 (28.10.96)	GB	9622366.4	28 October 1996 (28.10.96)	GB	9700699.3	15 January 1997 (15.01.97)	GB	9708265.5	24 April 1997 (24.04.97)	GB	9711842.6	6 June 1997 (06.06.97)	GB	9711845.9	6 June 1997 (06.06.97)	GB	<p>Anders [NO/NO]; Treskevn 32A, N-0681 Oslo (NO). TOLLESHAUG, Helge [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). GODAL, Aslak [NO/NO]; Nedre Silkestrå 16, N-0365 Oslo (NO). LØVHAUG, Dagfinn [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). SØLBAKKEN, Magne [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). CUTHERBERTSON, Alan [GB/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO).</p> <p>(74) Agent: <b>MARSDEN, John, Christopher; Frank B. Dehn &amp; Co., 179 Queen Victoria Street, London EC4V 4EL (GB).</b></p> <p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b></p>	
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<p><b>Published</b>  <i>Without international search report and to be republished upon receipt of that report.</i></p>																								
<p>(54) Title: <b>IMPROVEMENTS IN OR RELATING TO DIAGNOSTIC/THERAPEUTIC AGENTS</b></p> <p>(57) Abstract</p> <p>Targetable diagnostic and/or therapeutically active agents, e.g. ultrasound contrast agents, comprising a suspension in an aqueous carrier liquid of a reporter comprising gas-containing or gas-generating material, said reporter being conjugated to one or more non-proteinaceous, non-peptide and non-polysaccharide vectors.</p>																								

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Improvements in or relating to diagnostic/therapeutic agents

5        This invention relates to diagnostic and/or therapeutically active agents, more particularly to diagnostic and/or therapeutically active agents incorporating moieties which interact with or have affinity for sites and/or structures within the body so  
10      that diagnostic imaging and/or therapy of particular locations within the body may be enhanced. Of particular interest are diagnostic agents for use in ultrasound imaging, which are hereinafter referred to as targeted ultrasound contrast agents.

15      It is well known that ultrasound imaging comprises a potentially valuable diagnostic tool, for example in studies of the vascular system, particularly in cardiography, and of tissue microvasculature. A variety of contrast agents has been proposed to enhance the  
20      acoustic images so obtained, including suspensions of solid particles, emulsified liquid droplets, gas bubbles and encapsulated gases or liquids. It is generally accepted that low density contrast agents which are easily compressible are particularly efficient in terms  
25      of the acoustic backscatter they generate, and considerable interest has therefore been shown in the preparation of gas-containing and gas-generating systems.

30      Gas-containing contrast media are also known to be effective in magnetic resonance (MR) imaging, e.g. as susceptibility contrast agents which will act to reduce MR signal intensity. Oxygen-containing contrast media also represent potentially useful paramagnetic MR contrast agents.

35      Furthermore, in the field of X-ray imaging it has been observed that gases such as carbon dioxide may be used as negative oral contrast agents or intravascular

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contrast agents.

The use of radioactive gases, e.g. radioactive isotopes of inert gases such as xenon, has also been proposed in scintigraphy, for example for blood pool imaging.

Targeted ultrasound contrast agents may be regarded as comprising (i) a reporter moiety capable of interacting with ultrasound irradiation to generate a detectable signal; (ii) one or more vectors having affinity for particular target sites and/or structures within the body, e.g. for specific cells or areas of pathology; and (iii) one or more linkers connecting said reporter and vector(s), in the event that these are not directly joined.

The molecules and/or structure to which the agent is intended to bind will hereinafter be referred to as the target. In order to obtain specific imaging of or a therapeutic effect at a selected region/structure in the body the target must be present and available in this region/structure. Ideally it will be expressed only in the region of interest, but usually will also be present at other locations in the body, creating possible background problems. The target may either be a defined molecular species (i.e. a target molecule) or an unknown molecule or more complex structure (i.e. a target structure) which is present in the area to be imaged and/or treated, and is able to bind specifically or selectively to a given vector molecule.

The vector is attached or linked to the reporter moiety in order to bind these moieties to the region/structure to be imaged and/or treated. The vector may bind specifically to a chosen target, or it may bind only selectively, having affinity also for a limited number of other molecules/structures, again creating possible background problems.

There is a limited body of prior art relating to targeted ultrasound contrast agents. Thus, for example,

US-A-5531980 is directed to systems in which the reporter comprises an aqueous suspension of air or gas microbubbles stabilised by one or more film-forming surfactants present at least partially in lamellar or laminar form, said surfactant(s) being bound to one or more vectors comprising "bioactive species designed for specific targeting purposes". It is stated that the microbubbles are not directly encapsulated by surfactant material but rather that this is incorporated in liquid-filled liposomes which stabilise the microbubbles. It will be appreciated that lamellar or laminar surfactant material such as phospholipids present in such liposomes will inevitably be present in the form of one or more lipid bilayers with the lipophilic tails "back-to-back" and the hydrophilic heads both inside and outside (see e.g. Schneider, M. on "Liposomes as drug carriers: 10 years of research" in *Drug targeting, Nyon, Switzerland, 3-5 October 1984*, Buri, P. and Gumma, A. (Ed), Elsevier, Amsterdam 1984).

EP-A-0727225 describes targeted ultrasound contrast agents in which the reporter comprises a chemical having a sufficient vapour pressure such that a proportion of it is a gas at the body temperature of the subject. This chemical is associated with a surfactant or albumin carrier which includes a protein-, peptide- or carbohydrate-based cell adhesion molecule ligand as vector. The reporter moieties in such contrast agents correspond to the phase shift colloid systems described in WO-A-9416739; it is now recognised that administration of such phase shift colloids may lead to generation of microbubbles which grow uncontrollably, possibly to the extent where they cause potentially dangerous embolisation of, for example, the myocardial vasculature and brain (see e.g. Schwarz, *Advances in Echo-Contrast [1994(3)]*, pp 48-49).

WO-A-9320802 proposes that tissue-specific ultrasonic image enhancement may be achieved using

acoustically reflective oligolamellar liposomes conjugated to tissue-specific ligands such as antibodies, peptides, lectins etc. The liposomes are deliberately chosen to be devoid of gas and so will not have the advantageous echogenic properties of gas-based ultrasound contrast agents. Further references to this technology, e.g. in targeting to fibrin, thrombi and atherosclerotic areas are found in publications by Alkanonyuksel, H. et al. in *J. Pharm. Sci.* (1996) 85(5), 486-490; *J. Am. Coll. Cardiol.* (1996) 27(2) Suppl A, 298A; and *Circulation, 68 Sci. Sessions*, Anaheim 13-16 November 1995.

There is also a number of publications concerning ultrasound contrast agents which refer in passing to possible use of monoclonal antibodies as vectors without giving significant practical detail and/or to reporters comprising materials which may be taken up by the reticuloendothelial system and thereby permit image enhancement of organs such as the liver - see, for example WO-A-9300933, WO-A-9401140, WO-A-9408627, WO-A-9428874, US-A-5088499, US-A-5348016 and US-A-5469854.

The present invention is based on the finding that gas-containing and gas-generating diagnostic and/or therapeutic agents conjugated with certain non-proteinaceous, non-peptide and non-polysaccharide vectors are particularly useful targeting agents by virtue of their high degree of stability both *in vitro* and *in vivo*. Such products wherein the vectors are obtained from synthetic rather than natural sources will also avoid problems such as virus contamination and are advantageous in that the vector molecules may be clearly defined, thereby increasing product homogeneity and easy product documentation.

As used herein, the term "non-polysaccharide" means a chain of less than 200 sugar units, preferably less than 50 units, preferably comprising two or more different sugars. Even more preferred are sugar chains

comprising at least one substituent carrying amino sugar and even more preferred are branched sugar chains of a molecular weight of less than 5000.

One advantageous embodiment of the invention is based on the additional finding that limited adhesion to targets is a highly useful property of diagnostic and/or therapeutically active agents, which property may be achieved using vectors giving temporary retention rather than fixed adhesion to a target. Thus such agents, rather than being fixedly retained at specific sites, may for example effectively exhibit a form of retarded flow along the vascular endothelium by virtue of their transient interactions with endothelial cells. Such agents may thus become concentrated on the walls of blood vessels, in the case of ultrasound contrast agents providing enhanced echogenicity thereof relative to the bulk of the bloodstream, which is devoid of permanent structural features. They therefore may permit enhanced imaging of the capillary system, including the microvasculature, and so may facilitate distinction between normal and inadequately perfused tissue, e.g. in the heart, and may also be useful in visualising structures such as Kupffer cells, thrombi and atherosclerotic lesions or for visualising neovascularized and inflamed tissue areas. The present invention is particularly suited to imaging changes occurring in normal blood vessels situated in areas of tissue necrosis.

According to one aspect of the present invention there is provided a targetable diagnostic and/or therapeutic agent, e.g. an ultrasound contrast agent, comprising a suspension in an aqueous carrier liquid, e.g. an injectable carrier liquid, of a reporter comprising gas-containing or gas-generating material, said reporter being conjugated to one or more vectors, characterised in that said vectors are selected from non-polymeric synthetic or semi-synthetic vectors and

oligo/polynucleotides.

Vectors used in accordance with the invention are preferably exogenous to the human body.

The term "non-polymeric" as used herein in respect 5 of synthetic or semi-synthetic vectors is not intended to exclude oligomers.

Nucleotides used in accordance with the invention may, for example, contain 10-500 base units.

Oligonucleotides may, for example, contain 20-50 units, 10 whilst polynucleotides may, for example, contain 50-500 units.

In a further embodiment of the present invention, one or more vectors may be attached or included within the reporter in a manner such that the vectors are not 15 readily exposed to the target or target receptors.

Increased tissue specificity may therefore be achieved by applying an additional process to expose the vectors, for example by exposing the agent after administration to external ultrasound to modify the diffusibility of 20 the moieties containing the vectors.

Any biocompatible gas may be present in the reporter of contrast agents according to the invention, the term "gas" as used herein including any substances (including mixtures) substantially or completely in 25 gaseous (including vapour) form at the normal human body temperature of 37°C. The gas may thus, for example, comprise air; nitrogen; oxygen; carbon dioxide; hydrogen; an inert gas such as helium, argon, xenon or krypton; a sulphur fluoride such as sulphur 30 hexafluoride, disulphur decafluoride or trifluoromethylsulphur pentafluoride; selenium hexafluoride; an optionally halogenated silane such as methylsilane or dimethylsilane; a low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms), for 35 example an alkane such as methane, ethane, a propane, a butane or a pentane, a cycloalkane such as cyclopropane, cyclobutane or cyclopentane, an alkene such as ethylene,

propene, propadiene or a butene, or an alkyne such as acetylene or propyne; an ether such as dimethyl ether; a ketone; an ester; a halogenated low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms); or a mixture of any of the foregoing. Advantageously at least some of the halogen atoms in halogenated gases are fluorine atoms; thus biocompatible halogenated hydrocarbon gases may, for example, be selected from bromochlorodifluoromethane, chlorodifluoromethane, dichlorodifluoromethane, bromotrifluoromethane, chlorotrifluoromethane, chloropentafluoroethane, dichlorotetrafluoroethane, chlorotrifluoroethylene, fluoroethylene, ethylfluoride, 1,1-difluoroethane and perfluorocarbons, e.g. perfluoroalkanes such as perfluoromethane, perfluoroethane, perfluoropropanes, perfluorobutanes (e.g. perfluoro-n-butane, optionally in admixture with other isomers such as perfluoro-isobutane), perfluoropentanes, perfluorohexanes and perfluoroheptanes; perfluoroalkenes such as perfluoropropene, perfluorobutenes (e.g. perfluorobut-2-ene) and perfluorobutadiene; perfluoroalkynes such as perfluorobut-2-yne; and perfluorocycloalkanes such as perfluorocyclobutane, perfluoromethylcyclobutane, perfluorodimethylcyclobutanes, perfluorotrimethylcyclobutanes, perfluorocyclopentane, perfluoromethylcyclopentane, perfluorodimethylcyclopentanes, perfluorocyclohexane, perfluoromethylcyclohexane and perfluorocycloheptane. Other halogenated gases include methyl chloride, fluorinated (e.g. perfluorinated) ketones such as perfluoroacetone and fluorinated (e.g. perfluorinated) ethers such as perfluorodiethyl ether. The use of perfluorinated gases, for example sulphur hexafluoride and perfluorocarbons such as perfluoropropane, perfluorobutanes and perfluoropentanes, may be particularly advantageous in view of the recognised high stability in the bloodstream of microbubbles containing such gases.

The reporter may be in any convenient form, for example being any appropriate gas-containing or gas-generating ultrasound contrast agent formulation.

Representative examples of such formulations include

5 microbubbles of gas stabilised (e.g. at least partially encapsulated) by a coalescence-resistant surface membrane (for example gelatin, e.g. as described in WO-A-8002365), a filmogenic protein (for example an albumin such as human serum albumin, e.g. as described in US-A-10 4718433, US-A-4774958, US-A-4844882, EP-A-0359246, WO-A-9112823, WO-A-9205806, WO-A-9217213, WO-A-9406477 or WO-A-9501187), a polymer material (for example a synthetic biodegradable polymer as described in EP-A-0398935, an elastic interfacial synthetic polymer membrane as

15 described in EP-A-0458745, a microparticulate biodegradable polyaldehyde as described in EP-A-0441468, a microparticulate N-dicarboxylic acid derivative of a polyamino acid - polycyclic imide as described in EP-A-0458079, or a biodegradable polymer as described in WO-20 A-9317718 or WO-A-9607434), a non-polymeric and non-polymerisable wall-forming material (for example as described in WO-A-9521631), or a surfactant (for example a polyoxyethylene-polyoxypropylene block copolymer surfactant such as a Pluronic, a polymer surfactant as

25 described in WO-A-9506518, or a film-forming surfactant such as a phospholipid, e.g. as described in WO-A-9211873, WO-A-9217212, WO-A-9222247, WO-A-9428780 or WO-A-9503835).

30 Other useful gas-containing contrast agent formulations include gas-containing solid systems, for example microparticles (especially aggregates of microparticles) having gas contained therewithin or otherwise associated therewith (for example being adsorbed on the surface thereof and/or contained within voids, cavities or pores therein, e.g. as described in EP-A-0122624, EP-A-0123235, EP-A-0365467, WO-A-9221382,

WO-A-9300930, WO-A-9313802, WO-A-9313808 or WO-A-9313809). It will be appreciated that the echogenicity of such microparticulate contrast agents may derive directly from the contained/associated gas and/or from 5 gas (e.g. microbubbles) liberated from the solid material (e.g. upon dissolution of the microparticulate structure).

10 The disclosures of all of the above-described documents relating to gas-containing contrast agent formulations are incorporated herein by reference.

15 Gas microbubbles and other gas-containing materials such as microparticles preferably have an initial average size not exceeding 10 µm (e.g. of 7 µm or less) in order to permit their free passage through the pulmonary system following administration, e.g. by 20 intravenous injection.

Where phospholipid-containing compositions are employed in accordance with the invention, e.g. in the form of phospholipid-stabilised gas microbubbles, 25 representative examples of useful phospholipids include lecithins (i.e. phosphatidylcholines), for example natural lecithins such as egg yolk lecithin or soya bean lecithin and synthetic or semisynthetic lecithins such as dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine; phosphatidic acids; phosphatidylethanolamines; phosphatidylserines; phosphatidylglycerols; phosphatidylinositols; cardiolipins; sphingomyelins; fluorinated analogues of 30 any of the foregoing; mixtures of any of the foregoing and mixtures with other lipids such as cholesterol. The use of phospholipids predominantly (e.g. at least 75%) comprising molecules individually bearing net overall charge, e.g. negative charge, for example as in 35 naturally occurring (e.g. soya bean or egg yolk derived), semisynthetic (e.g. partially or fully hydrogenated) and synthetic phosphatidylserines,

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phosphatidylglycerols, phosphatidylinositols, phosphatidic acids and/or cardiolipins, may be particularly advantageous.

Additional exemplary lipids which may be used to 5 prepare gas-containing contrast agents include fatty acids, stearic acid, palmitic acid, 2-n-hexadecylstearic acid, oleic acid and other acid-containing lipid structures. Such lipid structures may be coupled by amide bond formation to amino acids containing one or 10 more amino groups; the resulting lipid-modified amino acids (e.g. dipalmitoyllysine or distearoyl-2,3-diaminopropionic acid) may be useful precursors for the attachment of functionalised spacer elements having coupling sites for conjugation of one or more vector 15 molecules.

Further useful stabilisers include lipopeptides comprising a lipid attached to a peptide portion which is suitably functionalised for coupling to one or more vector molecules. A particular preference is the 20 inclusion of a positively charged peptide linker element (e.g. comprising two or more lysine residues) capable of anchoring through electrostatic interaction with reporter microbubbles stabilised by negatively charged phospholipid or other surfactant membranes.

Another embodiment of the invention invention 25 comprises functionalised microbubbles carrying one or more reactive groups for non-specific reaction with a receptor molecules located on cell surfaces.

Microbubbles comprising a thiol moiety, for example, may 30 bind to cell surface receptors via disulphide exchange reactions. The reversible nature of such reactions means that microbubble flow may be controlled by altering the redox environment. Similarly, functionalised microbubbles with membranes comprising 35 activated esters such as N-hydroxysuccinimide esters may be used to react with amino groups found on a multiplicity of cell surface molecules.

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Representative examples of gas-containing microparticulate materials which may be useful in accordance with the invention include carbohydrates (for example hexoses such as glucose, fructose or galactose; 5 disaccharides such as sucrose, lactose or maltose; pentoses such as arabinose, xylose or ribose;  $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrins; polysaccharides such as starch, hydroxyethyl starch, amylose, amylopectin, glycogen, inulin, pulullan, dextran, carboxymethyl dextran, 10 dextran phosphate, ketodextran, aminoethyldextran, alginates, chitin, chitosan, hyaluronic acid or heparin; and sugar alcohols, including alditols such as mannitol or sorbitol), inorganic salts (e.g. sodium chloride), organic salts (e.g. sodium citrate, sodium acetate or 15 sodium tartrate), X-ray contrast agents (e.g. any of the commercially available carboxylic acid and non-ionic amide contrast agents typically containing at least one 2,4,6-triiodophenyl group having substituents such as carboxyl, carbamoyl, N-alkylcarbamoyl, N- 20 hydroxyalkylcarbamoyl, acylamino, N-alkylacylamino or acylaminomethyl at the 3- and/or 5-positions, as in metrizoic acid, diatrizoic acid, iothalamic acid, ioxaglic acid, iohexol, iopentol, iopamidol, iodixanol, iopromide, metrizamide, iodipamide, meglumine 25 iodipamide, meglumine acetrizoate and meglumine diatrizoate), and polypeptides and proteins (e.g. gelatin or albumin such as human serum albumin).

The reporter may be made by any convenient process, for example by making gas-containing or gas-generating formulations. Representative examples include 30 the preparation of a suspension of gas microbubbles by contacting a surfactant with gas and mixing them in the presence of an aqueous carrier, as described in WO 9115244; or by atomising a solution or dispersion of a 35 wall-forming material in the presence of a gas in order to obtain hollow microcapsules, as described in EP 512693A1; preparation of solid microspheres by a double

emulsion process, as described in US 5648095; or a process for forming hollow microcapsules by spray-drying as described in EP 681843A2; or preparing gas-filled liposomes by shaking an aqueous solution comprising a 5 lipid in the presence of a gas as described in US 5469854.

A suitable process for attachment of the desired vector to the reporter comprises a surface modification of the preformed reporter with a suitable linker 10 employing reactive groups on the surface of both the reporter and vector. It may be particularly advantageous physically to mix the reporter material with the vector-containing substance at any step of the process. Such a process will result in incorporation or an attachment of 15 the vector to the reporter. An optional process step may remove the excess of vector not bound to the reporter by washing the gas-containing particles following separation, by for example, floatation. A preferred aspect is the use of lipopeptide structures 20 incorporating functional groups such as thiol, maleimide biotin etc. which can be premixed if desired with other reporter molecules before formation of gas-containing agents. The attachment of vector molecules may be carried out using the linker reagents listed below.

Linking of a reporter unit to the desired vectors 25 may be achieved by covalent or non-covalent means, usually involving interaction with one or more functional groups located on the reporter and/or vectors. Examples of chemically reactive functional 30 groups which may be employed for this purpose include amino, hydroxyl, sulphhydryl, carboxyl, and carbonyl groups, as well as carbohydrate groups, vicinal diols, thioethers, 2-aminoalcohols, 2-aminothiols, guanidinyl, imidazolyl and phenolic groups.

Covalent coupling of reporter and vectors may 35 therefore be effected using linking agents containing reactive moieties capable of reaction with such

functional groups. Examples of reactive moieties capable of reaction with sulfhydryl groups include  $\alpha$ -haloacetyl compounds of the type X-CH<sub>2</sub>CO- (where X=Br, Cl or I), which show particular reactivity for sulfhydryl groups but which can also be used to modify imidazolyl, thioether, phenol and amino groups as described by Gurd, F.R.N. in *Methods Enzymol.* (1967) 11, 532. N-Maleimide derivatives are also considered selective towards sulfhydryl groups, but may additionally be useful in coupling to amino groups under certain conditions. N-maleimides may be incorporated into linking systems for reporter-vector conjugation as described by Kitagawa, T. et al. in *Chem. Pharm. Bull.* (1981) 29, 1130 or used as polymer crosslinkers for bubble stabilisation as described by Kovacic, P. et al. in *J. Am. Chem. Soc.* (1959) 81, 1887. Reagents such as 2-iminothiolane, e.g. as described by Traut, R. et al. in *Biochemistry* (1973) 12, 3266, which introduce a thiol group through conversion of an amino group, may be considered as sulfhydryl reagents if linking occurs through the formation of disulphide bridges. Thus reagents which introduce reactive disulphide bonds into either the reporter or the vector may be useful, since linking may be brought about by disulphide exchange between the vector and reporter; examples of such reagents include Ellman's reagent (DTNB), 4,4'-dithiodipyridine, methyl-3-nitro-2-pyridyl disulphide and methyl-2-pyridyl disulphide (described by Kimura, T. et al. in *Analyt. Biochem.* (1982) 122, 271).

Examples of reactive moieties capable of reaction with amino groups include alkylating and acylating agents. Representative alkylating agents include:

i)  $\alpha$ -haloacetyl compounds, which show specificity towards amino groups in the absence of reactive thiol groups and are of the type X-CH<sub>2</sub>CO- (where X=Cl, Br or I), e.g. as described by Wong, Y-H.H. in *Biochemistry* (1979) 24, 5337;

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ii) N-maleimide derivatives, which may react with amino groups either through a Michael type reaction or through acylation by addition to the ring carbonyl group as described by Smyth, D.G. et al. in *J. Am. Chem. Soc.* 5 (1960) 82, 4600 and *Biochem. J.* (1964) 91, 589;

iii) aryl halides such as reactive nitrohaloaromatic compounds;

iv) alkyl halides as described by McKenzie, J.A. et al. in *J. Protein Chem.* (1988) 7, 581;

v) aldehydes and ketones capable of Schiff's base formation with amino groups, the adducts formed usually being stabilised through reduction to give a stable amine;

vi) epoxide derivatives such as epichlorohydrin and bisoxiranes, which may react with amino, sulphydryl or phenolic hydroxyl groups;

vii) chlorine-containing derivatives of s-triazines, which are very reactive towards nucleophiles such as amino, sulphydryl and hydroxy groups;

viii) aziridines based on s-triazine compounds detailed above, e.g. as described by Ross, W.C.J. in *Adv. Cancer Res.* (1954) 2, 1, which react with nucleophiles such as amino groups by ring opening;

ix) squaric acid diethyl esters as described by Tietze, L.F. in *Chem. Ber.* (1991) 124, 1215; and

x)  $\alpha$ -haloalkyl ethers, which are more reactive alkylating agents than normal alkyl halides because of the activation caused by the ether oxygen atom, e.g. as described by Benneche, T. et al. in *Eur. J. Med. Chem.* 30 (1993) 28, 463.

Representative amino-reactive acylating agents include:

i) isocyanates and isothiocyanates, particularly aromatic derivatives, which form stable urea and thiourea derivatives respectively and have been used for protein crosslinking as described by Schick, A.F. et al. in *J. Biol. Chem.* (1961) 236, 2477;

ii) sulfonyl chlorides, which have been described by Herzig, D.J. et al. in *Biopolymers* (1964) 2, 349 and which may be useful for the introduction of a fluorescent reporter group into the linker;

5 iii) Acid halides;

iv) Active esters such as nitrophenylesters or N-hydroxysuccinimidyl esters;

v) acid anhydrides such as mixed, symmetrical or N-carboxyanhydrides;

10 vi) other useful reagents for amide bond formation as described by Bodansky, M. et al. in '*Principles of Peptide Synthesis*' (1984) Springer-Verlag;

vii) acylazides, e.g. wherein the azide group is generated from a preformed hydrazide derivative using sodium nitrite, e.g. as described by Wetz, K. et al. in *Anal. Biochem.* (1974) 58, 347;

15 viii) azlactones attached to polymers such as bis-acrylamide, e.g. as described by Rasmussen, J.K. in *Reactive Polymers* (1991) 16, 199; and

20 ix) Imidoesters , which form stable amidines on reaction with amino groups, e.g. as described by Hunter, M.J. and Ludwig, M.L. in *J. Am. Chem. Soc.* (1962) 84, 3491.

Carbonyl groups such as aldehyde functions may be reacted with weak bases. Weak bases include 1,2-aminothiols such as those found in N-terminal cysteine residues, which selectively form stable 5-membered thiazolidine rings with aldehyde groups, e.g. as described by Ratner, S. et al. in *J. Am. Chem. Soc.* (1937) 59, 200. Other weak bases such as phenyl hydrazones may be used, e.g. as described by Heitzman, H. et al. in *Proc. Natl. Acad. Sci. USA* (1974) 71, 3537.

Aldehydes and ketones may also be reacted with amines to form Schiff's bases, which may advantageously be stabilised through reductive amination. Alkoxylamino moieties readily react with ketones and aldehydes to produce stable alkoxamines, e.g. as

described by Webb, R. et al. in *Bioconjugate Chem.* (1990) 1, 96.

Examples of reactive moieties capable of reaction with carboxyl groups include diazo compounds such as 5 diazoacetate esters and diazoacetamides, which react with high specificity to generate ester groups, e.g. as described by Herriot R.M. in *Adv. Protein Chem.* (1947) 3, 169. Carboxylic acid modifying reagents such as carbodiimides, which react through O-acylurea formation 10 followed by amide bond formation, may also usefully be employed; linking may be facilitated through addition of an amine or may result in direct vector-receptor coupling. Useful water soluble carbodiimides include 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC) 15 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), e.g. as described by Zott, H.G. and Puett, D. in *J. Biol. Chem.* (1989) 264, 15552. Other useful carboxylic acid modifying reagents include isoxazolium derivatives such as Woodwards reagent K; chloroformates such as p-nitrophenylchloroformate; carbonyldiimidazoles such as 20 1,1'-carbonyldiimidazole; and N-carbalkoxydihydroquinolines such as N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.

Other potentially useful reactive moieties include 25 vicinal diones such as p-phenylenediglyoxal, which may be used to react with guanidinyl groups, e.g. as described by Wagner et al. in *Nucleic acid Res.* (1978) 5, 4065; and diazonium salts, which may undergo 30 electrophilic substitution reactions, e.g. as described by Ishizaka, K. and Ishizaka T. in *J. Immunol.* (1960) 85, 163. Bis-diazonium compounds are readily prepared by treatment of aryl diamines with sodium nitrite in acidic solutions. It will be appreciated that functional groups in the reporter and/or vector may if desired be 35 converted to other functional groups prior to reaction, e.g. to confer additional reactivity or selectivity. Examples of methods useful for this purpose include

conversion of amines to carboxylic acids using reagents such as dicarboxylic anhydrides; conversion of amines to thiols using reagents such as N-acetylhomocysteine thiolactone, S-acetylmercaptopsuccinic anhydride, 2-iminothiolane or thiol-containing succinimidyl derivatives; conversion of thiols to carboxylic acids using reagents such as  $\alpha$ -haloacetates; conversion of thiols to amines using reagents such as ethylenimine or 2-bromoethylamine; conversion of carboxylic acids to amines using reagents such as carbodiimides followed by diamines; and conversion of alcohols to thiols using reagents such as tosyl chloride followed by transesterification with thioacetate and hydrolysis to the thiol with sodium acetate.

Vector-reporter coupling may also be effected using enzymes as zero-length crosslinking agents; thus, for example, transglutaminase, peroxidase and xanthine oxidase have been used to produce crosslinked products. Reverse proteolysis may also be used for crosslinking through amide bond formation.

Non-covalent vector-reporter coupling may, for example, be effected by electrostatic charge interactions e.g. between a polylysinyl-functionalised reporter and a polyglutamyl-functionalised vector, through chelation in the form of stable metal complexes or through high affinity binding interaction such as avidin/biotin binding.

Alternatively, a vector may be coupled to a protein known to bind phospholipids. In many instances, a single molecule of phospholipid may attach to a protein such as a translocase, while other proteins may attach to surfaces consisting mainly of phospholipid head groups and so may be used to attach vectors to phospholipid microspheres; one example of such a protein is  $\beta$ 2-glycoprotein I (Chonn, A., Semple, S.C. and Cullis, P.R., *Journal of Biological Chemistry* (1995) 270, 25845-25849). Phosphatidylserine-binding proteins

have been described, e.g. by Igarashi, K. et al. in *Journal of Biological Chemistry* 270(49), 29075-29078. Annexins are a class of phospholipid-binding proteins, many of which bind particularly avidly to phosphatidylserine (reviewed in Raynal, P. and H.B. Pollard.

5 Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins". *Biochim. Biophys. Acta* 1197: 63-93). A conjugate of a vector with such a

10 phosphatidylserine-binding protein may therefore be used to attach the vector to phosphatidylserine-encapsulated microbubbles. When the amino acid sequence of a binding protein is known, the phospholipid-binding portion may be synthesised or isolated and used for conjugation with

15 a vector, thus avoiding the biological activity which may be located elsewhere in the molecule.

It is also possible to obtain molecules that bind specifically to the surface (or in the "membrane") of microspheres by direct screening of molecular libraries for microsphere-binding molecules. For example, libraries from combinatorial chemistry, displaying small molecules on the surface of small solid microspheres (about 0.02-2  $\mu\text{m}$ , preferably about 0.05-0.25  $\mu\text{m}$ ) could be used for such selection. The selection may be made

20 by simply mixing the gas-filled microspheres and the combinatorial library and eluting the small solid microspheres. - Alternatively, the combinatorial library could be produced on solid surfaces: the bottom of micro-titre wells. If desired, the selection may be

25 done under "physiological conditions" (e.g. in blood and/or under shear conditions) to eliminate peptides which cross-react with blood components. An advantage of this type of selection procedure is that only binding molecules that do not destabilize the gas-filled

30 microspheres should be selected, since only binding molecules attached to intact floating microspheres will rise to the top. It may also be possible to introduce some kind of "stress" during the selection procedure

(e.g. pressure) to ensure that destabilizing binding moieties are not selected. In this way it may be possible to select binders which may resist shear conditions present *in vivo*. A further advantage is the 5 option of selecting binding moieties with a limited affinity if permanent binding *in vivo* must be avoided. Binding moieties identified in this way may be coupled by chemical conjugation to a vector molecule, constituting a general tool for attaching any vector 10 molecule to the microspheres.

A vector which is coupled to a peptide, lipo-oligosaccharide or lipopeptide linker which contains an element capable of mediating membrane insertion may also be useful. One example is described by Leenhouts, J.M. 15 et al. in *Febs Letters* (1995) 370(3), 189-192. Non-bioactive molecules consisting of known membrane insertion anchor/signal groups may also be used as vectors for certain applications, an example being the H1 hydrophobic segment from the Na,K-ATPase  $\alpha$ -subunit 20 described by Xie, Y. and Morimoto, T. in *J. Biol. Chem.* (1995) 270(20), 11985-11991. The anchor group may also be fatty acid(s) or cholesterol.

Coupling may also be effected using avidin or streptavidin, which have four high affinity binding 25 sites for biotin. Avidin may therefore be used to conjugate vector to reporter if both vector and reporter are biotinylated. Examples are described by Bayer, E.A. and Wilchek, M. in *Methods Biochem. Anal.* (1980) 26, 1. This method may also be extended to include linking of 30 reporter to reporter, a process which may encourage bubble association and consequent potentially increased echogenicity. Alternatively, avidin or streptavidin may be attached directly to the surface of reporter microparticles.

35 Non-covalent coupling may also utilise the bifunctional nature of bispecific immunoglobulins. These molecules can specifically bind two antigens, thus

linking them. For example, either bispecific IgG or chemically engineered bispecific F(ab)'<sub>2</sub> fragments may be used as linking agents. Heterobifunctional bispecific antibodies have also been reported for linking two different antigens, e.g. as described by Bode, C. et al. in *J. Biol. Chem.* (1989) **264**, 944 and by Staerz, U.D. et al. in *Proc. Natl. Acad. Sci. USA* (1986) **83**, 1453. Similarly, any reporter and/or vector containing two or more antigenic determinants (e.g. as described by Chen, Aa et al. in *Am. J. Pathol.* (1988) **130**, 216) may be crosslinked by antibody molecules and lead to formation of multi-bubble cross-linked assemblies of potentially increased echogenicity.

Linking agents used in accordance with the invention will in general bring about linking of vector to reporter or reporter to reporter with some degree of specificity, and may also be used to attach one or more therapeutically active agents.

In some instances it is considered advantageous to include a PEG component as a stabiliser in conjunction with a vector or vectors or directly to the reporter in the same molecule where the PEG does not serve as a spacer.

Within the context of the present invention, the reporter unit will usually remain attached to the vectors. In another type of targeting procedure, sometimes called "pre-targeting", the vector (which may, for instance, be derivatized with a mono- or oligosaccharide) is administered alone; subsequently, the reporter is administered, coupled to a moiety which is capable of specifically binding the vector molecule (when the vector contains a sugar, the reporter may be coupled to a carbohydrate-binding molecule, such as a lectin). The advantage of this protocol is that time may be allowed for elimination of the vector molecules that do not bind their targets, substantially reducing the background problems that are connected with the presence

of an excess of reporter-vector conjugate. Within the context of the present invention, pre-targeting with one specific vector might be envisaged, followed by reporter units that are coupled to another vector and a moiety which binds the first vector.

Within the context of the present invention, in some cases and in particular for the assessment of blood perfusion rates in defined areas, for example in myocardium, it is of interest to measure the rate at which ultrasound contrast agents bound to the target are displaced or released from the target. This can be achieved in a controlled fashion by administration of the vector alone or other agents able to displace or release the ultrasound contrast agent from the target.

Ultrasound imaging modalities which may be used in accordance with the invention include two- and three-dimensional imaging techniques such as B-mode imaging (for example using the time-varying amplitude of the signal envelope generated from the fundamental frequency of the emitted ultrasound pulse, from sub-harmonics or higher harmonics thereof or from sum or difference frequencies derived from the emitted pulse and such harmonics, images generated from the fundamental frequency or the second harmonic thereof being preferred), colour Doppler imaging and Doppler amplitude imaging, and combinations of the two latter with any of the modalities (techniques) above. Surprisingly, the second harmonic signals from targeted monolayer microspheres were found to be excellent when used in accordance with the present invention. To reduce the effects of movement, successive images of tissues such as the heart or kidney may be collected with the aid of suitable

5 synchronisation techniques (e.g. gating to the ECG or respiratory movement of the subject). Measurement of changes in resonance frequency or frequency absorption which accompany arrested or retarded microbubbles may also usefully be made to detect the contrast agent.

The present invention provides a tool for therapeutic drug delivery in combination with vector-mediated direction of the product to the desired site. By "therapeutic" or "drug" is meant an agent having a 10 beneficial effect on a specific disease in a living human or non-human animal. Whilst combinations of drugs and ultrasound contrast agents have been proposed in, for example, WO-A-9428873 and WO-A-9507072, these products lack vectors having affinity for particular 15 sites and thereby show comparatively poor specific retention at desired sites prior to or during drug release.

Therapeutic compounds used in accordance with the present invention may be encapsulated in the interior of 20 the microbubbles/microparticles or attached to or incorporated into the structure thereof. Thus, the therapeutic compound may be linked to a part of the wall or matrix, for example through covalent or ionic bonds, or may be physically mixed into the encapsulating or 25 matrix material, particularly if the drug has similar polarity or solubility to this material, so as to prevent it from leaking out of the product before it is intended to act in the body. The release of the drug may be initiated merely by wetting contact with blood 30 following administration or as a consequence of other internal or external influences, e.g. dissolution processes catalyzed by enzymes or the use of of 35 ultrasound. The destruction of gas-containing microparticles using external ultrasound is a well known phenomenon in respect of ultrasound contrast agents, e.g. as described in WO-A-9325241; the rate of drug release may be varied depending on the type of

therapeutic application, using a specific amount of ultrasound energy from the transducer.

The therapeutic may be covalently linked to the membrane or matrix surface using a suitable linking agent, e.g. as described herein. Thus, for example, one may initially prepare a phospholipid or lipopeptide derivative to which the drug is bonded through a biodegradable bond or linker, and then incorporate this derivative into the material used to prepare the reporter, as described above. Alternatively, the product may initially be prepared without the therapeutic, which may then be coupled to or coated on the microbubbles or microparticles prior to use. Thus, for example, a therapeutic could be added to a suspension of microbubbles or microparticles in aqueous media and shaken in order to attach or adhere the therapeutic thereto.

Representative therapeutics suitable for use in the present drug delivery compositions include any known therapeutic drugs or active analogues thereof containing thiol groups which may couple to thiol-containing microbubbles under oxidative conditions yielding disulphide groups. In combination with a vector or vectors such drug/vector-modified microbubbles may be allowed to accumulate in target tissue. Administration of a reducing agent such as reduced glutathione then liberates the drug molecule from the targeted microbubble in the vicinity of the target cell, increasing the local concentration of the drug and enhancing its therapeutic effect. Alternatively the composition may initially be prepared without the therapeutic, which may then be coupled to or coated on the microbubbles immediately prior to use; thus, for example, a therapeutic could be added to a suspension of microbubbles in aqueous media and shaken in order to attach or adhere the therapeutic to the microbubbles.

Other drug delivery systems include vector-

modified phospholipid membranes doped with lipopeptide structures comprising a poly-L-lysine or poly-D-lysine chain in combination with a targeting vector. Applied to gene therapy/antisense technologies with particular emphasis on receptor-mediated drug delivery the microbubble carrier is condensed with DNA or RNA via electrostatic interaction with the cationic polylysine. This method has the advantage that the vector or vectors used for targeted delivery are not directly attached to the polylysine carrier moiety. The polylysine chain is also anchored more tightly in the microbubble membrane due to the presence of the lipid chains. The use of ultrasound to increase the effectiveness of delivery is also considered useful.

Alternatively free polylysine chains are firstly modified with drug or vector molecules then condensed onto the negative surface of targeted microbubbles.

Representative and non-limiting examples of drugs useful in accordance with the invention include antineoplastic agents such as vincristine, vinblastine, vindesine, busulfan, chlorambucil, spiroplatin, cisplatin, carboplatin, methotrexate, adriamycin, mitomycin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopurine, mitotane, procarbazine, dactinomycin (antinomycin D), daunorubicin, doxorubicin hydrochloride, taxol, plicamycin, aminoglutethimide, estramustine, flutamide, leuprolide, megestrol acetate, tamoxifen, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase), etoposide, interferon α-2a and 2b, blood products such as hematoporphyrins or derivatives of the foregoing; biological response modifiers such as muramylpeptides; antifungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine, miconazole or amphotericin B; hormones or hormone analogues such as growth hormone, melanocyte stimulating hormone, estradiol, beclomethasone dipropionate, betamethasone, cortisone acetate,

dexamethasone, flunisolide, hydrocortisone,  
methylprednisolone, paramethasone acetate, prednisolone,  
prednisone, triamcinolone or fludrocortisone acetate;  
vitamins such as cyanocobalamin or retinoids; enzymes  
5 such as alkaline phosphatase or manganese superoxide  
dismutase; antiallergic agents such as amelexanox;  
inhibitors of tissue factor such as monoclonal  
antibodies and Fab fragments thereof, synthetic  
peptides, nonpeptides and compounds downregulating  
10 tissue factor expression; inhibitors of platelets such  
as, GPIa, GPIb and GPIIb-IIIa, ADP receptors, thrombin  
receptors, von Willebrand factor, prostaglandins,  
aspirin, ticlopidin, clopigogrel and reopro; inhibitors  
15 of coagulation protein targets such as: FIIa FVa, FVIIa,  
FVIIIA, FIXa, tissue factor, heparins, hirudin, hirulog,  
argatroban, DEGR-rFVIIa and annexin V; inhibitors of  
fibrin formation and promoters of fibrinolysis such as  
t-PA, urokinase, Plamin, Streptokinase, rt-Plasminogen  
Activator and rStaphylokinase; antiangiogenic factors  
20 such as medroxyprogesterone, pentosan polysulphate,  
suramin, taxol, thalidomide, angiostatin, interferon-  
alpha, metalloproteinase inhibitors, platelet factor 4,  
somatostatin, thrombospondin; circulatory drugs such as  
propranolol; metabolic potentiators such as glutathione;  
25 antituberculars such as p-aminosalicylic acid,  
isoniazid, capreomycin sulfate, cycloserine, ethambutol,  
ethionamide, pyrazinamide, rifampin or streptomycin  
sulphate; antivirals such as acyclovir, amantadine,  
azidothymidine, ribavirin or vidarabine; blood vessel  
30 dilating agents such as diltiazem, nifedipine,  
verapamil, erythritol tetranitrate, isosorbide  
dinitrate, nitroglycerin or pentaerythritol  
tetranitrate; antibiotics such as dapsone,  
chloramphenicol, neomycin, cefaclor, cefadroxil,  
35 cephalixin, cephadrine, erythromycin, clindamycin,  
lincomycin, amoxicillin, ampicillin, bacampicillin,  
carbenicillin, dicloxacillin, cyclacillin,

picloxacillin, hetacillin, methicillin, nafcillin, penicillin, polymyxin or tetracycline; antiinflammatories such as diflunisal, ibuprofen, indomethacin, meclofenamate, mefenamic acid, naproxen, 5 phenylbutazone, piroxicam, tolmetin, aspirin or salicylates; antiprotozoans such as chloroquine, metronidazole, quinine or meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric; opiates such as codeine, morphine or opium; 10 cardiac glycosides such as deslaneside, digitoxin, digoxin, digitalin or digitalis; neuromuscular blockers such as atracurium mesylate, gallamine triethiodide, hexafluorenium bromide, metocurine iodide, pancuronium bromide, succinylcholine chloride, tubocurarine chloride 15 or vecuronium bromide; sedatives such as amobarbital, amobarbital sodium, apropbarbital, butabarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotrimeprazine hydrochloride, methyprylon, midazolam hydrochloride, 20 paraldehyde, pentobarbital, secobarbital sodium, talbutal, temazepam or triazolam; local anaesthetics such as bupivacaine, chloroprocaine, etidocaine, lidocaine, mepivacaine, procaine or tetracaine; general anaesthetics such as droperidol, etomidate, fentanyl 25 citrate with droperidol, ketamine hydrochloride, methohexitol sodium or thiopental and pharmaceutically acceptable salts (e.g. acid addition salts such as the hydrochloride or hydrobromide or base salts such as sodium, calcium or magnesium salts) or derivatives (e.g. acetates) thereof. Other examples of therapeutics include genetic material such as nucleic acids, RNA, and DNA of natural or synthetic origin, including recombinant RNA and DNA. DNA encoding certain proteins may be used in the treatment of many different types of 30 diseases. For example, tumor necrosis factor or interleukin-2 genes may be provided to treat advanced cancers; thymidine kinase genes may be provided to treat 35

ovarian cancer or brain tumors; interleukin-2 genes may be provided to treat neuroblastoma, malignant melanoma or kidney cancer; and interleukin-4 genes may be provided to treat cancer.

5        Lipophilic derivatives of drugs linked to microbubble membranes through hydrophobic interactions may exhibit therapeutic effects as part of the microbubble or after release from the microbubble, e.g. by use of ultrasound. If the drug does not possess the  
10      desired physical properties, a lipophilic group may be introduced for anchoring the drug to the membrane. Preferably the lipophilic group should be introduced in a way that does not influence the *in vivo* potency of the molecule, or the lipophilic group may be cleaved  
15      releasing the active drug. Lipophilic groups may be introduced by various chemical means depending on functional groups available in the drug molecule. Covalent coupling may be effected using functional groups in the drug molecule capable of reacting with  
20      appropriately functionalised lipophilic compounds. Examples of lipophilic moieties include branched and unbranched alkyl chains, cyclic compounds, aromatic residues and fused aromatic and non-aromatic cyclic systems. In some instances the lipophilic moiety will consist of a suitably functionalised steroid, like cholesterol and related compounds. Examples of  
25      functional groups particularly suitable for derivatisation include nucleophilic groups like amino, hydroxy and sulfhydryl groups. Suitable processes for  
30      lipophilic derivatisation of any drug containing a sulfhydryl group, like captopril, may include direct alkylation, e.g. reaction with an alkyl halide under basic conditions and thiol ester formation by reaction with an activated carboxylic acid. Representative  
35      examples of derivatisation of any drug having carboxylic functions, like atenolol and chlorambucil, include amide and ester formation by coupling of amines and alcohols,

respectively, possessing appropriate physical properties. A preferred embodiment comprises attachment of cholesterol to a therapeutic compound by forming a degradable ester bond.

5       A preferred application of the present invention relates to angiogenesis, which is the formation of new blood vessels by branching from existing vessels. The primary stimulus for this process may be inadequate supply of nutrients and oxygen (hypoxia) to cells in a  
10      tissue. The cells may respond by secreting angiogenetic factors, of which there are many; one example is *vascular endothelial growth factor*. These factors initiate the secretion of proteolytic enzymes which break down the proteins of the basement membrane, as  
15      well as inhibitors which limit the action of these potentially harmful enzymes. The combined effect of loss of attachment and signals from the receptors for angiogenetic factors is to cause the endothelial cells to move, multiply, and rearrange themselves, and finally  
20      to synthetise a basement membrane around the new vessels.

25      Tumors must initiate angiogenesis when they reach millimeter size in order to keep up their rate of growth. As angiogenesis is accompanied by characteristic changes in the endothelial cells and their environment, this process is a promising target for therapeutic intervention.

30      Many recent disclosures describe non-peptidic compounds useful for this purpose. The transformations accompanying angiogenesis are very promising for diagnosis, a preferred example being malignant disease, but the concept also shows great promise in inflammation and a variety of inflammation-related diseases. These factors are also involved in re-vascularisation of  
35      infarcted parts of the myocardium, which occurs if a stenosis is released within a short time.

A number of known receptors/targets associated

with angiogenesis are given in subsequent tables. Using the targeting principles described in the present disclosure, angiogenesis may be detected by the majority of the imaging modalities in use in medicine.

5      Contrast-enhanced ultrasound may possess additional advantages, the contrast medium being microspheres which are restricted to the interior of blood vessels. Even if the target antigens are found on many cell types, the microspheres will attach exclusively to endothelial  
10     cells.

So-called prodrugs may also be used in agents according to the invention. Thus drugs may be derivatised to alter their physicochemical properties and to adapt them for inclusion into the reporter; such  
15     derivatised drugs may be regarded as prodrugs and are usually inactive until cleavage of the derivatising group regenerates the active form of the drug.

By targeting a gas-filled microbubble containing a prodrug-activating enzyme to areas of pathology one may  
20     image targeting of the enzyme, making it possible to visualise when the micobubbles are targeted properly to the area of pathology and at the same time have disappeared from non-target areas. In this way one can determine the optimal time for injection of prodrug  
25     into individual patients.

Another alternative is to incorporate the prodrug, the prodrug-activating enzyme and the vector in the same microbubble in a system where the prodrug will only be activated after some external stimulus. Such a stimulus  
30     may, for example, be a tumour-specific protease as described above, or bursting of the bubbles by external ultrasound after the desired targeting has been achieved.

35      Therapeutics may easily be delivered in accordance with the invention to diseased or necrotic areas including the heart and vasculature in general, and to

the liver, spleen and kidneys and other regions such as the lymph system, body cavities or gastrointestinal system.

Products according to the present invention may be used for targeted therapeutic delivery either *in vivo* or *in vitro*. In the latter context the products may be useful in *in vitro* systems such as kits for diagnosis of different diseases or characterisation of different components in blood or tissue samples. Similar techniques to those used to attach certain blood components or cells to polymer particles (e.g. monodisperse magnetic particles) *in vitro* to separate them from a sample may be used in the present invention, using the low density of the reporter units in agents of the present invention to effect separation of the gas-containing material by floatation and repeated washing.

So-called zero-length linking agents, which induce direct covalent joining of two reactive chemical groups without introducing additional linking material (e.g. as in amide bond formation induced using carbodiimides or enzymatically) may, if desired, be used in accordance with the invention, as may agents such as biotin/avidin systems which induce non-covalent reporter-vector linking and agents which induce hydrophobic or electrostatic interactions.

Most commonly, however, the linking agent will comprise two or more reactive moieties, e.g. as described above, connected by a spacer element. The presence of such a spacer permits bifunctional linkers to react with specific functional groups within a molecule or between two different molecules, resulting in a bond between these two components and introducing extrinsic linker-derived material into the reporter-vector conjugate. The reactive moieties in a linking agent may be the same (homobifunctional agents) or different (heterobifunctional agents) or, where several

dissimilar reactive moieties are present, heteromultifunctional agents), providing a diversity of potential reagents that may bring about covalent bonding between any chemical species, either intramolecularly or 5 intermolecularly.

The nature of extrinsic material introduced by the linking agent may have a critical bearing on the targeting ability and general stability of the ultimate product. Thus it may be desirable to introduce labile 10 linkages, e.g. by using spacers which are biodegradable or chemically sensitive or which incorporate enzymatic cleavage sites. Alternatively the spacer may include polymeric components, e.g. to act as surfactants and enhance bubble stability. The spacer may also contain 15 reactive moieties, e.g. as described above to enhance surface crosslinking, or it may contain a tracer element such as a fluorescent probe, spin label or radioactive material.

Contrast agents according to the present invention 20 are therefore useful in all imaging modalities since contrast elements such as X-ray contrast agents, light imaging probes, spin labels or radioactive units may readily be incorporated in or attached to the reporter units.

Spacer elements may typically consist of aliphatic chains which effectively separate the reactive moieties of the linker by distances of between 5 and 30 Å. They 25 may also comprise macromolecular structures such as poly(ethylene glycols). Such polymeric structures, hereinafter referred to as PEGs, are simple, neutral polyethers which have been given much attention in biotechnical and biomedical applications (see e.g. Milton Harris, J. (ed) "Poly(ethylene glycol) chemistry, biotechnical and biomedical applications" Plenum Press, 30 New York, 1992). PEGs are soluble in most solvents, including water, and are highly hydrated in aqueous environments, with two or three water molecules bound to 35

each ethylene glycol segment; this has the effect of preventing adsorption either of other polymers or of proteins onto PEG-modified surfaces. PEGs are known to be nontoxic and not to harm active proteins or cells,  
5 whilst covalently linked PEGs are known to be non-immunogenic and non-antigenic. Furthermore, PEGs may readily be modified and bound to other molecules with only little effect on their chemistry. Their advantageous solubility and biological properties are  
10 apparent from the many possible uses of PEGs and copolymers thereof, including block copolymers such as PEG-polyurethanes and PEG-polypropylenes.

Appropriate molecular weights for PEG spacers used in accordance with the invention may, for example, be  
15 between 120 Daltons and 20 kDaltons.

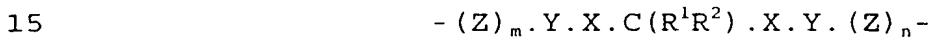
The major mechanism for uptake of particles by the cells of the reticuloendothelial system (RES) is opsonisation by plasma proteins in blood; these mark foreign particles which are then taken up by the RES.  
20 The biological properties of PEG spacer elements used in accordance with the invention may serve to increase contrast agent circulation time in a similar manner to that observed for PEGylated liposomes (see e.g. Klibanov, A.L. et al. in *FEBS Letters* (1990) 268, 235-  
25 237 and Blume, G. and Cevc, G. in *Biochim. Biophys. Acta* (1990) 1029, 91-97). Increased coupling efficiency to areas of interest may also be achieved using antibodies bound to the terminii of PEG spacers (see e.g. Maruyama, K. et al. in *Biochim. Biophys. Acta* (1995) 1234, 74-80 and Hansen, C.B. et al. in *Biochim. Biophys. Acta* (1995) 1239, 133-144).

In some instances it is considered advantageous to include a PEG component as a stabiliser in conjunction with a vector or vectors or directly to the reporter in  
35 the same molecule where the PEG does not serve as a spacer.

Other representative spacer elements include

structural-type polysaccharides such as polygalacturonic acid, glycosaminoglycans, heparinoids, cellulose and marine polysaccharides such as alginates, chitosans and carrageenans; storage-type polysaccharides such as 5 starch, glycogen, dextran and aminodextrans; polyamino acids and methyl and ethyl esters thereof, as in homo- and co-polymers of lysine, glutamic acid and aspartic acid; and polypeptides and oligosaccharides, which may or may not contain enzyme cleavage sites.

10 In general, spacer elements may contain cleavable groups such as vicinal glycol, azo, sulfone, ester, thioester or disulphide groups. Spacers containing biodegradable methylene diester or diamide groups of formula



[where X and Z are selected from -O-, -S-, and -NR- (where R is hydrogen or an organic group); each Y is a carbonyl, thiocarbonyl, sulphonyl, phosphoryl or similar acid-forming group: m and n are each zero or 1; and R<sup>1</sup> and R<sup>2</sup> are each hydrogen, an organic group or a group -X.Y.(Z)<sub>m</sub>-, or together form a divalent organic group] may also be useful; as discussed in, for example, WO-A-9217436 such groups are readily biodegraded in the presence of esterases, e.g. *in vivo*, but are stable in 20 the absence of such enzymes. They may therefore advantageously be linked to therapeutic agents to permit 25 slow release thereof.

Poly[N-(2-hydroxyethyl)methacrylamides] are potentially useful spacer materials by virtue of their 30 low degree of interaction with cells and tissues (see e.g. Volfová, I., Ríhová, B. and V.R. and Vetvicka, P. in *J. Bioact. Comp. Polymers* (1992) 7, 175-190). Work on a similar polymer consisting mainly of the closely related 2-hydroxypropyl derivative showed that it was 35 endocytosed by the mononuclear phagocyte system only to a rather low extent (see Goddard, P., Williamson, I., Bron, J., Hutchinson, L.E., Nicholls, J. and Petrak, K.

in *J. Biost. Compat. Polym.* (1991) 6, 4-24.).

Other potentially useful polymeric spacer materials include:

- i) copolymers of methyl methacrylate with methacrylic acid; these may be erodible (see Lee, P.I. in *Pharm. Res.* (1993) 10, 980) and the carboxylate substituents may cause a higher degree of swelling than with neutral polymers;
- 10 ii) block copolymers of polymethacrylates with biodegradable polyesters (see e.g. San Roman, J. and Guillen-Garcia, P. in *Biomaterials* (1991) 12, 236-241);
- iii) cyanoacrylates, i.e. polymers of esters of 2-cyanoacrylic acid - these are biodegradable and have been used in the form of nanoparticles for selective 15 drug delivery (see Forestier, F., Gerrier, P., Chaumard, C., Quero, A.M., Couvreur, P. and Labarre, C. in *J. Antimicrob. Chemoter.* (1992) 30, 173-179);
- iv) polyvinyl alcohols, which are water-soluble and generally regarded as biocompatible (see e.g. Langer, R. 20 in *J. Control. Release* (1991) 16, 53-60);
- v) copolymers of vinyl methyl ether with maleic anhydride, which have been stated to be bioerodible (see Finne, U., Hannus, M. and Urtti, A. in *Int. J. Pharm.* (1992) 78, 237-241);
- 25 vi) polyvinylpyrrolidones, e.g. with molecular weight less than about 25,000, which are rapidly filtered by the kidneys (see Hespe, W., Meier, A. M. and Blankwater, Y. M. in *Arzeim.-Forsch./Drug Res.* (1977) 27, 1158-1162);
- 30 vii) polymers and copolymers of short-chain aliphatic hydroxyacids such as glycolic, lactic, butyric, valeric and caproic acids (see e.g. Carli, F. in *Chim. Ind. (Milan)* (1993) 75, 494-9), including copolymers which incorporate aromatic hydroxyacids in order to increase 35 their degradation rate (see Imasaki, K., Yoshida, M., Fukuzaki, H., Asano, M., Kumakura, M., Mashimo, T., Yamanaka, H. and Nagai. T. in *Int. J. Pharm.* (1992) 81,

31-38);

viii) polyesters consisting of alternating units of ethylene glycol and terephthalic acid, e.g. Dacron<sup>R</sup>, which are non-degradable but highly biocompatible;

5 ix) block copolymers comprising biodegradable segments of aliphatic hydroxyacid polymers (see e.g. Younes, H., Nataf, P.R., Cohn, D., Appelbaum, Y.J., Pizov, G. and Uretzky, G. in *Biomater. Artif. Cells Artif. Organs* (1988) **16**, 705-719), for instance in conjunction with

10 polyurethanes (see Kobayashi, H., Hyon, S.H. and Ikada, Y. in "Water-curable and biodegradable prepolymers" - *J. Biomed. Mater. Res.* (1991) **25**, 1481-1494);

15 x) polyurethanes, which are known to be well-tolerated in implants, and which may be combined with flexible "soft" segments, e.g. comprising poly(tetra methylene glycol), poly(propylene glycol) or poly(ethylene glycol)) and aromatic "hard" segments, e.g. comprising 4,4'-methylenebis(phenylene isocyanate) (see e.g. Ratner, B.D., Johnston, A.B. and Lenk, T.J. in

20 *J. Biomed. Mater. Res: Applied Biomaterials* (1987) **21**, 59-90; Sa Da Costa, V. et al. in *J. Coll. Interface Sci.* (1981) **80**, 445-452 and Affrossman, S. et al. in *Clinical Materials* (1991) **8**, 25-31);

25 xi) poly(1,4-dioxan-2-ones), which may be regarded as biodegradable esters in view of their hydrolysable ester linkages (see e.g. Song, C. X., Cui, X. M. and Schindler, A. in *Med. Biol. Eng. Comput.* (1993) **31**, S147-150), and which may include glycolide units to improve their absorbability (see Bezwada, R.S., Shalaby, S.W. and Newman, H.D.J. in *Agricultural and synthetic polymers: Biodegradability and utilization* (1990) (ed Glass, J.E. and Swift, G.), 167-174 - ACS symposium Series, #433, Washington D.C., U.S.A. - American Chemical Society);

30 xii) polyanhydrides such as copolymers of sebacic acid (octanedioic acid) with bis(4-carboxy-phenoxy)propane, which have been shown in rabbit studies (see Brem, H.,

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Kader, A., Epstein, J.I., Tamargo, R.J., Domb, A.,  
Langer, R. and Leong, K.W. in *Sel. Cancer Ther.* (1989)  
5, 55-65) and rat studies (see Tamargo, R.J., Epstein,  
J.I., Reinhard, C.S., Chasin, M. and Brem, H. in *J.  
Biomed. Mater. Res.* (1989) 23, 253-266) to be useful for  
controlled release of drugs in the brain without evident  
toxic effects;  
xiii) biodegradable polymers containing ortho-ester  
groups, which have been employed for controlled release  
10 *in vivo* (see Maa, Y.F. and Heller, J. in *J. Control.  
Release* (1990) 14, 21-28); and  
xiv) polyphosphazenes, which are inorganic polymers  
consisting of alternate phosphorus and nitrogen atoms  
(see Crommen, J.H., Vandorpe, J. and Schacht, E.H. in *J.  
Control. Release* (1993) 24, 167-180).  
15

The following tables list linking agents and  
agents for protein modification which may be useful in  
preparing targetable agents in accordance with the  
invention.

20

Heterobifunctional linking agents

	<b>Linking agent</b>	<b>Reactivity 1</b>	<b>Reactivity 2</b>	<b>Comments</b>
5	ABH	carbohydrate	photoreactive	
	ANB-NOS	-NH <sub>2</sub>	photoreactive	
	APDP(1)	-SH	photoreactive	iodinable disulphide linker
	APG	-NH <sub>2</sub>	photoreactive	reacts selectively with Arg at pH 7-8
	ASIB(1)	-SH	photoreactive	iodinable
	ASBA(1)	-COOH	photoreactive	iodinable
10	EDC	-NH <sub>2</sub>	-COOH	zero-length linker
	GMBS	-NH <sub>2</sub>	-SH	
	sulfo-GMBS	-NH <sub>2</sub>	-SH	water-soluble
	HSAB	-NH <sub>2</sub>	photoreactive	
	sulfo-HSAB	-NH <sub>2</sub>	photoreactive	water-soluble
	MBS	-NH <sub>2</sub>	-SH	
15	sulfo-MBS	-NH <sub>2</sub>	-SH	water-soluble
	M <sub>2</sub> C <sub>2</sub> H	carbohydrate	-SH	
	MPBH	carbohydrate	-SH	
	NHS-ASA(1)	-NH <sub>2</sub>	photoreactive	iodinable
	sulfo-NHS-ASA(1)	-NH <sub>2</sub>	photoreactive	water-soluble, iodinable
	sulfo-NHS-LC-ASA(1)	-NH <sub>2</sub>	photoreactive	water-soluble, iodinable
20	PDPH	carbohydrate	-SH	disulphide linker
	PNP-DTP	-NH <sub>2</sub>	photoreactive	

	SADP	-NH <sub>2</sub>	photoreactive	disulphide linker
5	sulfo-SADP	-NH <sub>2</sub>	photoreactive	water-soluble disulphide linker
	SAED	-NH <sub>2</sub>	photoreactive	disulphide linker
	SAND	-NH <sub>2</sub>	photoreactive	water-soluble disulphide linker
	SANPAH	-NH <sub>2</sub>	photoreactive	
	sulfo-SANPAH	-NH <sub>2</sub>	photoreactive	water-soluble
	SASD(1)	-NH <sub>2</sub>	photoreactive	water-soluble iodinable disulphide linker
	SIAB	-NH <sub>2</sub>	-SH	
10	sulfo-SIAB	-NH <sub>2</sub>	-SH	water-soluble
	SMCC	-NH <sub>2</sub>	-SH	
	sulfo-SMCC	-NH <sub>2</sub>	-SH	water-soluble
	SMPB	-NH <sub>2</sub>	-SH	
	sulfo-SMPB	-NH <sub>2</sub>	-SH	water-soluble
	SMPT	-NH <sub>2</sub>	-SH	
15	sulfo-LC-SMPT	-NH <sub>2</sub>	-SH	water-soluble
	SPDP	-NH <sub>2</sub>	-SH	
	sulfo-SPDP	-NH <sub>2</sub>	-SH	water-soluble
	sulfo-LC-SPDP	-NH <sub>2</sub>	-SH	water-soluble
	sulfo-SAMCA(2)	-NH <sub>2</sub>	photoreactive	
20	sulfo-SAPB	-NH <sub>2</sub>	photoreactive	water-soluble

Notes: (1)=iodinable; (2)=fluorescent

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Homobifunctional linking agents

	<b>Linking agent</b>	<b>Reactivity</b>	<b>Comments</b>
5	BS	-NH <sub>2</sub>	
	BMH	-SH	
	BASED(1)	photoreactive	iodinable disulphide linker
	BSCOES	-NH <sub>2</sub>	
	sulfo-BSCOES	-NH <sub>2</sub>	water-soluble
	DFDNB	-NH <sub>2</sub>	
10	DMA	-NH <sub>2</sub>	
	DMP	-NH <sub>2</sub>	
	DMS	-NH <sub>2</sub>	
	DPDPB	-SH	disulphide linker
	DSG	-NH <sub>2</sub>	
	DSP	-NH <sub>2</sub>	disulphide linker
15	DSS	-NH <sub>2</sub>	
	DST	-NH <sub>2</sub>	
	sulfo-DST	-NH <sub>2</sub>	water-soluble
	DTBP	-NH <sub>2</sub>	disulphide linker
	DTSSP	-NH <sub>2</sub>	disulphide linker
	EGS	-NH <sub>2</sub>	
20	sulfo-EGS	-NH <sub>2</sub>	water-soluble
	SPBP	-NH <sub>2</sub>	
	(1: iodinable)		

25

Biotinylation agents

	<b>Agent</b>	<b>Reactivity</b>	<b>Comments</b>
30	biotin-BMCC	-SH	
	biotin-DPPE		preparation of biotinylated liposomes

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	biotin-LC-DPPE		preparation of biotinylated liposomes
	biotin-HPDP	-SH	disulphide linker
	biotin-hydrazide	carbohydrate	
	biotin-LC-hydrazide	carbohydrate	
5	iodoacetyl-LC-biotin	-NH <sub>2</sub>	
	NHS-iminobiotin	-NH <sub>2</sub>	reduced affinity for avidin
	NHS-SS-biotin	-NH <sub>2</sub>	disulphide linker
	photoactivatable biotin	nucleic acids	
	sulfo-NHS-biotin	-NH <sub>2</sub>	water-soluble
10	sulfo-NHS-LC-biotin	-NH <sub>2</sub>	

Notes: DPPE=dipalmitoylphosphatidylethanolamine; LC=long chain

#### Agents for protein modification

15

Agent	Reactivity	Function
Ellman's reagent	-SH	quantifies/detects/protects
DTT	-S.S-	reduction
2-mercaptoethanol	-S.S-	reduction
20 2-mercaptoethylamine	-S.S-	reduction
Traut's reagent	-NH <sub>2</sub>	introduces -SH
SATA	-NH <sub>2</sub>	introduces protected -SH
AMCA-NHS	-NH <sub>2</sub>	fluorescent labelling
AMCA-hydrazide	carbohydrate	fluorescent labelling
25 AMCA-HPDP	-S.S-	fluorescent labelling
SBF-chloride	-S.S-	fluorescent detection of -SH
N-ethylmaleimide	-S.S-	blocks -SH
NHS-acetate	-NH <sub>2</sub>	blocks and acetylates -NH <sub>2</sub>
citraconic anhydride	-NH <sub>2</sub>	reversibly blocks and introduces negative charges

DTPA	-NH <sub>2</sub>	introduces chelator
BNPS-skatole	tryptophan	cleaves tryptophan residue
Bolton-Hunter	-NH <sub>2</sub>	introduces iodinable group

5           Other potentially useful protein modifications include partial or complete deglycosidation by glycosidases, neuraminidase, endoglycosyldases or periodate, since deglycosidation often results in less uptake by liver, spleen, macrophages etc., whereas neo-  
 10          glycosylation of proteins often results in increased uptake by the liver and macrophages); preparation of truncated forms by proteolytic cleavage, leading to reduced size and shorter half life in circulation; and cationisation, e.g. as described by Kumagi *et al.* in *J. Biol. Chem.* (1987) **262**, 15214-15219; Triguero *et al.* in *Proc. Natl. Acad. Sci. USA* (1989) **86**, 4761-4765; Pardridge *et al.* in *J. Pharmacol. Exp. Therap.* (1989) **251**, 821-826 and Pardridge and Boado, *Febs Lett.* (1991) **288**, 30-32.

20          Vectors which may be usefully employed in targetable agents according to the invention include the following, where appropriate in the form of non-bioactive analogues:

25          i)       Non-peptide agonists/antagonists or binders of receptors for cell adhesion molecules, cytokines, growth factors and peptide hormones. This category may include non-bioactive vectors which will be neither agonists nor antagonist but which may nonetheless exhibit valuable targeting ability.

30          ii)      Oligonucleotides and modified oligonucleotides which bind DNA or RNA through Watson-Crick or other types of base-pairing. DNA is usually only present in extracellular space as a consequence of cell damage, so that such oligonucleotides, which will usually be non-

bioactive, may be useful in, for example, targeting of necrotic regions, which are associated with many different pathological conditions. Oligonucleotides may also be designed to bind to specific DNA- or RNA-binding 5 proteins, for example transcription factors which are very often highly overexpressed or activated in tumour cells or in activated immune or endothelial cells. Combinatorial libraries may be used to select oligonucleotides which bind specifically to any possible 10 target molecules (from the examples of proteins to caffeine) and which therefore may be employed as vectors for targeting.

iii) DNA-binding drugs may behave similarly to 15 oligonucleotides, but may exhibit biological activity and/or toxic effects if taken up by cells.

iv) Protease substrates/inhibitors. Proteases are involved in many pathological conditions and the 20 substrates/inhibitors of such proteases are often non-peptidic. Some low molecular weight protease substrates and inhibitors are known to be non-bioactive.

v) Non-peptide vector molecules, e.g aptamers 25 (nucleic acid molecules) may be generated from combinatorial libraries without necessarily knowing the exact molecular target, by functionally selecting (in vitro, ex vivo or in vivo) for molecules binding to the region/structure, to be imaged. The target structure can 30 be sugars, lipids, peptides, proteins or nucleic acids.

vi) Various small molecules, including bioactive 35 compounds known to bind to biological receptors of various kinds. Such vectors or their targets may be used to generate non-bioactive compounds binding to the same targets.

The following tables identify various vectors which may be targeted to particular types of targets and indicated areas of use for targetable diagnostic and/or therapeutic agents according to the invention which 5 contain such vectors. It will be appreciated that where appropriate non-bioactive analogues of such vectors should be employed.

10 Vectors comprising non-peptide agonists/antagonists or non-bioactive binders of receptors for cytokines/growth factors/peptide hormones/cell adhesion molecules

	<b>Vector type</b>	<b>Target</b>	<b>Comments/areas of use</b>	<b>Ref</b>
15	Endothelin antagonist	Endothelin receptor	Vessel wall	34
20	Angiotensin II antagonists CV-11974, TCV-116	Angiotensin II receptors	Vessel wall brain adrenal gland	
	non-peptide RGD-analogues	integrins	Cells in immune system vessel wall etc.	5

25

Oligonucleotide vectors

	<b>Vector type</b>	<b>Target</b>	<b>Comments/areas of use</b>	<b>Ref</b>
30	Oligonucleotides complementary to repeated sequences, e.g. genes for ribosomal RNA, Alu-sequences	DNA made available by necrosis	Tumours Myocardial infarction All other diseases that involves necrosis	6
35				

	Oligonucleotides complementary to disease-specific mutations (e.g. mutated oncogenes).	DNA made available by necrosis in a region of the relevant disease	Tumours	6
5	Oligonucleotides complementary to DNA of infecting agent.	DNA of infective agent	Viral or bacterial infections	6
10	Triple or quadruple-helix forming oligonucleotides	As in above examples	As in above examples	6
15	Oligonucleotides with recognition sequence for DNA- or RNA- binding proteins	DNA-binding protein, e.g. transcription factors (often overexpressed/ activated in tumours or activated endothelium/ immune cells	Tumours Activated endothelium Activated immune cells	
20	Aptamers = Oligonucleotides complementary to any sequence. Selected from comb. libraries.	Protein- or peptide sequence, sugars, lipids, nucleic acids	Tumours Activated endothelium Activated immune cells Any disease-specific structure	
25				

Modified oligonucleotide vectors (e.g. modified to increase stability in vivo)

	Vector type	Target	Comments/areas of use	Ref
5	Phosphorothioate oligos	As for unmodified oligos	As for unmodified oligos	6
	2'-O-methyl substituted oligos	"	"	6
10	circular oligos	"	"	6
	oligos containing hairpin structure to decrease degradation	"	"	6
15	oligos with terminal phosphorothioate	"	"	6
20	2'-fluoro oligos	"	"	6
	2'-amino oligos	"	"	6
25	DNA-binding drugs conjugated to oligos (for examples, see below)	"	Increased binding affinity as compared to pure oligos	7
30	Peptide Nucleic Acids (PNAs, oligonucleotidss with a peptide backbone)	"	Increased binding affinity and stability compared to standard oligos.	8

Nucleoside and nucleotide vectors

<b>Vector type</b>	<b>Target</b>	<b>Comments/areas of use</b>	<b>Ref</b>
5 Adenosine and analogues	Adenosine receptors	Vessel wall Heart	9
	ATP, ADP, UDP, UTP and others	Various nucleotide receptors	10 Many tissues, e.g. brain, spinal cord, kidney, spleen

10 Receptors comprising DNA-binding drugs

<b>Vector type</b>	<b>Target</b>	<b>Comments/areas of use</b>	<b>Ref</b>
15 acridine derivatives distamycin netropsin actinomycin D echinomycin bleomycin etc.	DNA made available by necrosis	Tumours, Myocardial infarction and all other diseases involving necrosis or other processes liberating DNA from cells	

20

Receptors comprising protease substrates

<b>Vector type</b>	<b>Target</b>	<b>Comments/areas of use</b>	<b>Ref</b>
25 Non-peptidic substrates	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	11
	Metallo-proteinases	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds	

30

Receptors comprising protease inhibitors

Vector type	Target	Comments/areas of use	Ref
5 3,4-dichloroiso-coumarin [Fln]N-[N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl]agmatine	Cathepsin B	Tumours, a variety of which may more or less specifically overexpress proteases of various kinds, e.g. Cathepsin B	11
10 N- $\alpha$ -tosyl-L-lysine chloromethyl ketone			
15 bestatin ([(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanoyl]-L-leucine hydrochloride)	Aminopeptidases	Tumours, e.g. on cell surfaces	
20 Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)	Serine proteases	Tumours, vessel wall etc.	
25 Commercially available inhibitors e.g. kaptopril enalapril rictionopril	Angiotensin converting enzyme	Endothelial cells	
30 Low specificity non-peptidic compounds	Coagulation factors	Vessel wall injury, tumours, etc.	

Vectors comprising anti-angiogenic factors

Vector type	Target	Comments/areas of use	Ref
AGM-1470	EC of tumors	Kaposi's sarcoma, malignant tumors	D
5 Amiloride	uPA	Neoplasms	A
Fumagillin and analogs	EC of tumors		D
Genistein	EC of tumors	Neoplasms	E
10 Medroxyprogesterone	EC of tumors		K
Mitoflaxone	EC of tumors	Solid tumors	D
Pentosan polysulfate	EC of tumors		K
Raloxifene-HCl	EC of tumors	Solid tumors	K, D
15 Suramin	EC of tumors	Prostate carcinoma	K
Taxol	EC of tumors		K
Tamoxifen	EC of tumors	Neoplasms	E
Tecogalan	EC of tumors	Kaposi's sarcoma, malignant tumors	D
Thalidomide	EC of tumors		K
20 EC - endothelial cells			

Vectors comprising angiogenic factors

Vector type	Target	Comments/areas of use	Ref
25 Prostaglandins E1, E2	EC of tumors		K
Platelet activating factor	EC of tumors		K
Heparin	EC of tumors		K
30 Hyaluronic acid or fragments thereof	EC of tumors		K
Adenosine	EC of tumors		K

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Nicotinamide	EC of tumors		K
Histamine	EC of tumors		K
Spermine	EC of tumors		K

5

Vectors from combinatorial libraries

	<b>Vector type</b>	<b>Target</b>	<b>Comments/areas of use</b>	<b>Ref</b>
10	Oligonucleotides with sequence determined during generation process	Coagulation factors	Vessel wall injury, tumours etc.	12, 13, 14
15	Modifications of oligos obtained as above	"	"	12, 13, 14
20	Other chemicals with structure determined during generation process	"	"	12, 13, 14

25

Non-polymeric carbohydrate vectors

	<b>Vector type</b>	<b>Target</b>	<b>Comments/areas of use</b>	<b>Ref</b>
30	various	liver	liver diseases	
	oligosaccharides with terminal galactose	Asialo-glycoprotein receptor		15

35

Small molecule vectors

Vector type	Target	Comments/areas of use	Ref
Adrenalin	Corresponding receptors		
5 Betablockers	Adrenergic beta-receptors	Myocardium for beta-1 blockers	
Alpha-blockers	Adrenergic alpha-receptors	Vessel wall	
benzodiazepines			
serotonin-analogues	Serotonin-receptors		
10 anti-histamines	Histamine-receptors	Vessel wall	
Acetyl-choline receptor antagonists	ACh-receptors		
verapamil	Ca <sup>2+</sup> -channel blocker	Heart muscle	
15 Amiloride	Na'/H'-exchanger	Blocks this exchanges in kidney and is generally upregulated in cells stimulated by growth factors.	
Thromboxane/ Prostaglandin receptor antagonists or agonists	Thromboxane/ prostaglandin receptors	Vessel wall, Endothelium	
20			

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	Non-peptide hormones/neuro- transmitters , analogues/ antagonists or other drugs acting through cell surface receptors or on extracellular targets		
5			
10	Biotin	biotin transport protein on cell surface	17
15	Folate	folate transport protein on cell surface	Tumours 18
	Riboflavin	riboflavin transport protein on cell surface	19

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The following non-limitative examples serve to illustrate the invention. Confirmation of the microparticulate nature of products is performed using 10 microscopy as described in WO-A-9607434. Ultrasonic transmission measurements may be made using a broadband transducer to indicate suspensions of products giving an increased sound beam attenuation compared to a standard. Flow cytometric analysis of products can be used to 15 confirm attachment of molecules thereto. The ability of targeted agents to bind specifically to cells expressing a target may be studied by microscopy and/or using a flow chamber containing immobilised cells, for example employing a population of cells expressing the target 20 structure and a further population of cells not expressing the target. Radioactive or fluorescent or enzyme-labelled streptavidin/avidin may be used to analyse biotin attachment.

Example 1 - Gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotin-amidocaproate-PEG<sub>3400</sub>-Ala-cholesterol

5      a) Synthesis of Z-Ala-cholesterol (3-O-(carbobenzyloxy-L-alanyl)cholesterol)

Cholesterol (4 mmol), Z-alanine (5 mmol) and dimethylaminopyridine (4 mmol) were dissolved in 10 dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and dicyclohexylcarbodiimide was added. The reaction mixture was stirred at ambient temperature overnight. Dicyclohexylurea was filtered off and the solvent was rotary evaporated. The residue was taken up in 15 chloroform, undissolved dicyclohexylurea was filtered off and the solvent was removed by rotary evaporation. The residue was placed on a column of silica gel, and Z-Ala-cholesterol was eluted with toluene/petroleum ether (20:2) followed by toluene/diethyl ether (20:2). The 20 fractions containing the title compound were combined and the solvent was removed by rotary evaporation. The structure of the product was confirmed by NMR.

25      b) Synthesis of Ala-cholesterol (3-O-(L-alanyl)-cholesterol)

Z-Ala-cholesterol (0.48 mmol) is placed in 30 tetrahydrofuran (20 ml) and glacial acetic acid (3 ml) and hydrogenated in the presence of 5 % palladium on charcoal for 2 hours. The reaction mixture is filtered and concentrated *in vacuo*.

c) Synthesis of Boc-NH-PEG<sub>3400</sub>-Ala-cholesterol

35      Ala-cholesterol is added to a solution of Boc-NH-PEG<sub>3400</sub>-SC (t-butyl carbamate poly(ethylene glycol)-succinimidyl carbonate) (Shearwater) in chloroform,

followed by triethylamine. The suspension is stirred at 41 °C for 10 minutes. The crude product is purified by chromatography.

5       d) Synthesis of H<sub>2</sub>N-PEG<sub>3400</sub>-Ala-cholesterol

Boc-NH-PEG<sub>3400</sub>-Ala-cholesterol is stirred in 4 M hydrochloric acid in dioxane for 2.5 hours at ambient temperature. The solvent is removed by rotary evaporation and the residue is taken up in chloroform and washed with water. The organic phase is rotary evaporated to dryness. The crude product may be purified by chromatography.

15      e) Synthesis of biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol

A solution of biotinamidocaproate N-hydroxysuccinimide ester in tetrahydrofuran is added to H<sub>2</sub>N-PEG<sub>3400</sub>-Ala-cholesterol dissolved in tetrahydrofuran and 0.1 M sodium phosphate buffer having a pH of 7.5 (2 ml). The reaction mixture is heated to 30 °C and the reaction is followed to completion by TLC, whereafter the solvent is evaporated.

25

f) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol

30      To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine (in total 90-99.9mol%) and biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol (10-0.1mol%) is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is then transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The

vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated.

5

g) Alternative preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol

10

To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is then transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water. Biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol dissolved in water is added the washed microbubbles, which are placed on a roller table for several hours. The washing procedure is repeated following incorporation of the biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol into the microbubble membranes.

Example 2 - Gas-containing microparticles comprising phosphatidylserine, phosphatidylcholine, biotinamidocaproate-PEG<sub>3400</sub>-Ala-Cholesterol and drug-cholesterol

30

a) Synthesis of drug-cholesterol

Cholesterol (4mmol), a drug having an acid group (see Example 4(b) for a list of cholesterol-derivatised drugs) and dimethylaminopyridine (4 mmol) are dissolved in dimethylformamide/tetrahydrofuran (20 ml + 5 ml) and dicyclohexylcarbodiimide is added. The reaction mixture

is stirred at ambient temperature overnight. Dicyclohexylurea is filtered off and the solvent is rotary evaporated. The title compound is purified by chromatography.

5

b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine, biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol and drug-cholesterol

10

To a mixture (5 mg) of phosphatidylserine and phosphatidylcholine (in total 90-99.9mol%) and biotinamidocaproate-PEG<sub>3400</sub>-Ala-cholesterol (prepared as in Example 1) and drug-cholesterol (in total 10-0.1mol%)

15 is added 5% propyleneglycol-glycerol in water (1 ml).

The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The

20 vial is shaken in a cap-mixer for 45 seconds whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated.

25

Example 3 - Biotin attached to gas-filled microbubbles

Biotin may be attached to microbubbles in many different ways, e.g. in a similar way to that described by Corley, P. and Loughrey, H.C. in (1994) *Biochim. Biophys. Acta*

30 1195, 149-156. The resulting bubbles are analysed by flow cytometry, e.g. by employing fluorescent streptavidin to detect attachment of biotin to the bubbles. Alternatively radioactive or enzyme-labelled streptavidin/avidin is used to analyse biotin attachment.

35

Example 4 - Gas-filled microbubbles encapsulated with

- 59 -

1.2-distearoyl-sn-Glycero-3-[Phospho-L-Serine] and  
biotin-DPPE

1.2-distearoyl-sn-Glycero-3-[Phospho-L-Serine] (Avanti  
5 lot# 180PS-12, 22.6 mg) was added 4% propylenglycol-  
glycerol in water (4 ml). The dispersion was heated, to  
not more than 80 °C for five minutes, and then cooled to  
ambient temperature. An aqueous dispersion of biotin-  
DPPE (Pierce lot# 96092472, 1.5 mg) in 4%  
10 propylenglycol-glycerol (1 ml) was added and the sample  
was put on a roller table for 1-2 hours. The suspension  
was filled on vials and head spaces were flushed with  
perfluorobutane. The vials were shaken for 45 seconds  
whereafter they were put on a roller table. After  
15 centrifugation for seven minutes the infranatant was  
exchanged with water and the washing was repeated two  
times.

Normal Phase HPLC with an Evaporative Light Scattering  
20 Detector confirmed that the membranes of the  
microbubbles contained 4 mol% biotin-DPPE. The mean  
particle diameter of the microbubbles was 4 µm measured  
by Coulter Counter. Ultrasound transmission measurements  
using a 3.5 MHz broadband transducer showed that a  
25 particle dispersion of < 2 mg/ml gave a sound beam  
attenuation higher than 5 dB/cm.

Example 5 - Gas-filled microbubbles encapsulated with  
phosphatidylserine and biotinylated vector non-  
30 covalently bound to streptavidin-Succ-PEG-DSPE

a) Synthesis of Succ-PEG<sub>3400</sub>-DSPE

NH<sub>2</sub>-PEG<sub>3400</sub>-DSPE (prepared as in Preparation 1) is  
35 carboxylated using succinic anhydride, e.g. by a similar  
method to that described by Nayar, R. and Schroit, A.J.  
in *Biochemistry* (1985) 24, 5967-71.

b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and Succ-PEG<sub>3400</sub>-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%) and Succ-PEG<sub>3400</sub>-DSPE (10-0.1 mol%) is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes and then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation the infranatant is exchanged with water and the washing is repeated. Alternatively the microbubbles may be prepared as described in Preparation 1(f).

c) Coupling of streptavidin to gas-filled microbubbles encapsulated with phosphatidylserine and Succ-PEG<sub>3400</sub>-DSPE

Streptavidin is covalently bound to Succ-PEG<sub>3400</sub>-DSPE in the membrane by standard coupling methods using a water-soluble carbodiimide. The sample is placed on a roller table during the reaction. After centrifugation the infranatant is exchanged with water and the washing is repeated. The functionality of the attached streptavidin is analysed by binding, e.g. to fluorescently labeled biotin, biotinylated antibodies (detected with a fluorescently labeled secondary antibody) or biotinylated and fluorescence- or radioactively-labeled oligonucleotides. Analysis is performed by fluorescence microscopy or scintillation counting.

d) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and biotin non-covalently bound to streptavidin-Succ-PEG<sub>3400</sub>-DSPE

Microbubbles from (c) above are incubated in a solution containing biotinylated vectors, e.g. biotinylated drugs, carbohydrates, modified oligos, inhibitors etc. The vector-coated microbubbles are washed as described  
5 above.

Example 6 - Gas-filled microbubbles encapsulated with phosphatidylserine and biotinylated oligonucleotide non-covalently bound to streptavidin-Succ-PEG-DSPE

10

a) Synthesis of Succ-PEG<sub>3400</sub>-DSPE

NH<sub>2</sub>-PEG<sub>3400</sub>DSPE (prepared as in Preparation 1) is carboxylated using succinic anhydride, e.g. by a similar  
15 method to that described by Nayar, R. and Schroit, A.J. in *Biochemistry* (1985) 24, 5967-71.

20

b) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and Succ-PEG<sub>3400</sub>-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%) and Succ-PEG<sub>3400</sub>-DSPE (10-0.1 mol%) is added 5% propyleneglycol-glycerol in water (1 ml). The dispersion is heated to not more than 80 °C for 5 minutes  
25 and then cooled to ambient temperature. The dispersion (0.8 ml) is transferred to a vial (1 ml) and the head space is flushed with perfluorobutane. The vial is shaken in a cap-mixer for 45 seconds, whereafter the sample is put on a roller table. After centrifugation  
30 the infranatant is exchanged with water and the washing is repeated. Alternatively the microbubbles may be prepared as described in Preparation 1(f).

c) Coupling of streptavidin to gas-filled microbubbles encapsulated with phosphatidylserine and Succ-PEG<sub>3400</sub>-DSPE

Streptavidin is covalently bound to succ-PEG<sub>3400</sub>-DSPE in

the microbubbles by standard coupling methods using a water soluble carbodiimide. The sample is placed on a roller table during the reaction. After centrifugation the infranatant is exchanged with water and the washing 5 is repeated. The functionality of the attached streptavidin is analysed by binding, e.g. to fluorescently labeled biotin, biotinylated antibodies (detected with a fluorescently labeled secondary antibody) or biotinylated and fluorescence- or 10 radioactively-labeled oligonucleotides. Analysis is performed by fluorescence microscopy or scintillation counting.

15 d) Preparation of gas-filled microbubbles encapsulated with phosphatidylserine and a biotinylated oligonucleotide non-covalently bound to streptavidin-succ-PEG<sub>3400</sub>-DSPE

Microbubbles from (c) above are incubated in a solution 20 containing a biotinylated oligonucleotide. The oligonucleotide-coated bubbles are washed as described above. Binding of the oligonucleotide to the bubbles is detected e.g. by using fluorescent-labeled oligonucleotides for attachment to the bubbles, or by hybridising the attached oligonucleotide to a labeled 25 (fluorescence or radioactivity) complementary oligonucleotide. The functionality of the oligonucleotide-carrying microbubbles is analysed, e.g. by hybridising the bubbles with immobilized DNA- 30 containing sequences complementary to the attached oligonucleotide. As examples, an oligonucleotide complementary to ribosomal DNA (of which there are many copies per haploid genome) and an oligonucleotide complementary to an oncogene (e.g. ras of which there is 35 one copy per haploid genome) are used.

Example 7 - Gas-filled microbubbles encapsulated with

phosphatidylserine and folate-PEG-Succ-DSPEa) Preparation of folate-PEG-Succ-DSPE

5 Folate-PEG-Succ-DSPE is synthesised as described by Lee,  
R.J. and Low, P.S. in (1995) *Biochimica. Biophysica.*  
*Acta* 1233, 134-144.

b) Preparation of gas-filled microbubbles encapsulated  
10 with phosphatidylserine and folate-PEG-Succ-DSPE

To a mixture (5 mg) of phosphatidylserine (90-99.9 mol%)  
and Folate-PEG-DSPE (10-0.1 mol%) is added 5%  
propyleneglycol-glycerol in water (1 ml). The  
15 dispersion is heated to not more than 80 °C for 5 minutes  
and is then cooled to ambient temperature. The  
dispersion (0.8 ml) is transferred to a vial (1 ml) and  
the head space is flushed with perfluorobutane. The vial  
is shaken in a cap-mixer for 45 seconds, whereafter the  
20 sample is put on a roller table. After centrifugation  
the infranatant is exchanged with water and the washing  
is repeated. Alternatively the microbubbles are  
prepared as described in Preparation 1(e) or (f).  
Analysis of folate attachment may for example be done by  
25 microscopic study of the binding of the folate-  
containing microbubbles to cells expressing different  
levels of folate receptors.

Example 8 - Gas-containing microparticles comprising  
30 polymer from ethylidene bis(16-hydroxyhexadecanoate) and  
adipoyl chloride and biotin-amidocaproate-Ala covalently  
attached to the polymera) Synthesis of Z-Ala-polymer (3-O-(carbobenzyloxy-L-  
35 alanyl)-polymer)

The polymer is prepared from ethylidene bis(16-

hydroxyhexadecanoate) and adipoyl chloride as described in WO-A-9607434, and a polymer fraction with molecular weight 10000 is purified using gel permeation chromatography (GPC). 10 g of the material  
5 (corresponding to 1 mmol OH groups), Z-alanine (5 mmol) and dimethylaminopyridine (4 mmol) are dissolved in dry dimethylformamide/tetrahydrofuran and dicyclohexylcarbodiimide is then added. The reaction mixture is stirred at ambient temperature overnight.  
10 Dicyclohexylurea is filtered off and the solvent is removed using rotary evaporation. The product is purified by chromatography, fractions containing the title compound are combined and the solvent is removed using rotary evaporation. The structure of the product  
15 is confirmed by NMR.

b) Synthesis of Ala-polymer (3-O-(L-alanyl)-polymer)

Z-Ala-polymer (0.1 mmol) is stirred in  
20 toluene/tetrahydrofuran and glacial acetic acid (15% of the total volume) and hydrogenated in the presence of 5 % palladium on charcoal for 2 hours. The reaction mixture is filtered and concentrated *in vacuo*.

25 c) Synthesis of biotinamidocaproate-Ala-polymer

A solution of biotinamidocaproate N-hydroxysuccinimide ester in tetrahydrofuran is added to  
H<sub>2</sub>N-Ala-polymer dissolved in a mixture of tetrahydrofuran  
30 and dimethylformamide and 0.1 M sodium phosphate buffer having a pH of 7.5. The reaction mixture is heated to 30 °C and stirred vigorously; the reaction is followed by TLC to completion. The solvent is evaporated and the crude product is used without further purification.

35

d) Gas-containing particles comprising biotin-amidocaproate-Ala-polymer and PEG 10000 methyl ether 16-

hexadecanoyloxyhexadecanoate

10 ml of a 5% w/w solution of biotin-amidocaproate-Ala-polymer in (-)-camphene maintained at 60 °C is added to  
5 30 ml of an 1% w/w aqueous solution of PEG 10000 methyl ether 16-hexadecanoyloxyhexadecanoate (prepared as described in WO-A-9607434) at the same temperature. The mixture is emulsified using a rotor stator mixer (Ultra Turax® T25) at a slow speed for several minutes, and  
10 thereafter is frozen in a dry ice/methanol bath and lyophilized for 48 hours, giving the title product as a white powder.

15 e) Acoustic characterisation and microscopy of the product

Confirmation of the microparticulate nature of the product is performed using light microscopy as described in WO-A-9607434. Ultrasonic transmission measurements  
20 using a 3.5 MHz broadband transducer indicate that a particle suspension of < 2 mg/ml gives a sound beam attenuation of at least 5 dB/cm.

25 Example 9 - Gas-filled microbubbles encapsulated with phosphatidylserine, phosphatidylcholine and 3β-[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol

Monolayer-encapsulated microbubbles containing perfluorobutane are made from a mixture of 10 %  
30 phosphatidylserine, 50-80 % phosphatidylcholine (PC) and 10-40 % 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-chol) (Farhood, H., Gao, X., Barsoum, J. and Huang, L., *Anal. Biochem.* 225, 89-93 (1995)).

35

Example 10 - Functionalisation of gas-filled albumin microspheres (GAM) with biotin

A homogeneous suspension of GAM ( $6 \times 10^8$  particles/ml) in 5 mg/ml albumin was used, with all manipulations being carried out at room temperature. Two 10 ml aliquots were centrifuged (170 x g, 5 minutes) to promote 5 flotation of the microspheres and 8 ml of the underlying infranatant was removed by careful suction and replaced by an equal volume of air-saturated phosphate buffered saline, the preparations being rotated for 15-20 minutes to resuspend the microspheres. This procedure was 10 repeated twice, whereafter only negligible amounts of free non-microsphere-associated albumin were assumed to remain.

15 50  $\mu$ l of NHS-biotin (10 mM in dimethylsulphoxide) was added to one of the aliquots (final concentration 50  $\mu$ M); the other (control) aliquot received 50  $\mu$ l of dimethylsulphoxide. The tubes containing the samples were rotated for 1 hour whereafter 20  $\mu$ l portions of 50% aqueous glutaraldehyde were added to each tube to 20 crosslink the microspheres. After rotation for another hour the tubes were positioned vertically overnight to allow flotation of the microspheres. The next day, the suspensions were washed twice with phosphate buffered saline containing 1 mg/ml human serum albumin (PBS/HSA) 25 and were resuspended in PBS/HSA after the last centrifugation.

In order to determine the presence of microsphere-associated biotin, streptavidin conjugated to 30 horseradish peroxidase (strep-HRP) was added to both suspensions and the tubes were rotated for 1 hour to allow for reaction. The microspheres were then washed three times, resuspended in 100 mM citrate-phosphate buffer (pH 5) containing 0.1 mg/ml phenylenediamine 35 dihydrochloride and 0.01% hydrogen peroxide, and rotated for 10 minutes. Development of a yellow-green colour was indicative of the presence of enzyme. The following

results were obtained:

<u>Sample</u>	<u>Colour development</u>
5      Biotinylated spheres + strp-HRP	2+
Control spheres + strp-HRP	+

This confirms that GAM were biotinylated.

10

Example 11 - Gas-filled microbubbles encapsulated with distearoylphosphatidylserine comprising a captopril-containing molecule for diagnostic therapeutic applications

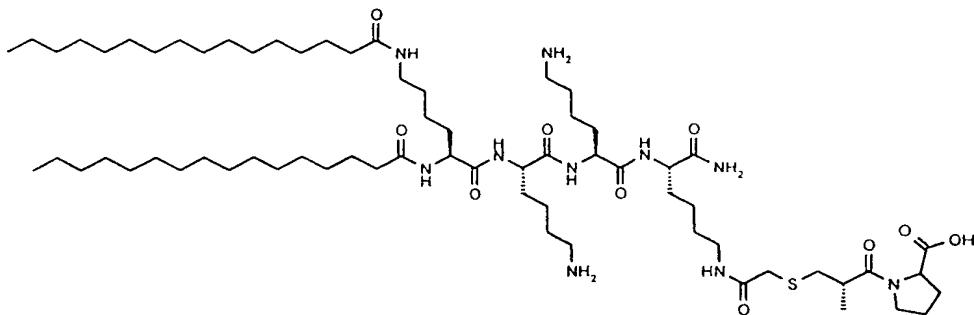
15

a) Synthesis of a lipopeptide functionalised with captopril:

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25

30



The structure shown above was synthesised by the manual bubbler method starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale. All amino acids were purchased from Novabiochem and palmitic acid from Fluka. Coupling was carried out using standard TBTU/HOBt/DIEA protocol. Bromoacetic acid was coupled through the side-chain of Lys as a symmetrical anhydride using DIC preactivation. Captopril (Sigma) dissolved in DMF was introduced on the solid phase using DBU as base. Simultaneous removal of the peptide from the resin and

deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT, 5% water and 5% ethyl methyl sulphide for 2 h. An aliquot of 10 mg of the crude material was purified by preparative liquid chromatography (Vydac 218TP1022 column) using a gradient of 70 to 100% B over 60 min (A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 ml/min. After lyophilisation a yield of 2 mg of pure material was obtained (analytical HPLC: gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile; flow rate 1 ml/min; column Vydac 218TP54; detection UV 214 nm; retention time 26 min). Further characterisation was carried out using MALDI mass spectrometry, giving M+H at 1265 as expected.

15

b) Preparation of gas-containing microbubbles of distearoyl-phosphatidyl-serine comprising a compound containing captopril

20 A solution of 1.4% propylene glycol/2.4% glycerol (1.0 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) and product from a) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then warmed to 80 °C for 5 min (vial was shaken during 25 warming). The vial was cooled and the head space was flushed with perfluorobutane gas. The vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no detectable level of compound from a) in the final wash 30 solution.

Incorporation of captopril containing lipopeptide into the bubbles was confirmed by MALDI mass spectrometry as follows. Ca 50 µl of microbubbles were transferred to a 35 clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI mass spectrometry, giving a M+H peak corresponding to the

lipopeptide from a).

5           Example 12. Gas-filled microbubbles encapsulated with phosphatidyl-serine comprising a vector with affinity for adrenergic receptors for diagnostic and therapeutic applications

10           a) Synthesis of a protected atenolol derivative suitable for solid phase coupling

15           i) Synthesis of methyl 4-[(2,3-epoxy)propoxy]phenylacetate

20           A mixture of methyl 4-hydroxyphenylacetate (4.98 g, 0.030 mol), epichlorohydrin (23.5 ml, 0.30 mol) and pyridine (121  $\mu$ l, 1.5 mmol) was stirred at 85 °C for 2 h. The reaction mixture was cooled, and excess epichlorohydrin was distilled off (rotavapor). The residue was taken up in ethyl acetate, washed with brine and dried ( $\text{Na}_2\text{SO}_4$ ). The solution was filtered and concentrated. The dark residue was chromatographed (silica, hexane/ethyl acetate 7:3) to give 2.25 g (34%) of a colourless oil.  $^1\text{H}$  (300 MHz) and  $^{13}\text{C}$  NMR (75 MHz) spectra were in accordance with the structure.

25           ii) Synthesis of methyl 4-[2-hydroxy-3-[(1-methyl-ethyl)amino]propoxy]phenylacetate

30           A mixture of methyl 4-[(2,3-epoxy)propoxy]phenylacetate (2.00 g, 9.00 mmol), isopropylamine (23 ml, 0.27 mol) and water (1.35 ml, 74.7 mmol) was stirred at room temperature overnight. The reaction mixture was concentrated (rotavapor) and the oily residue was dissolved in chloroform and dried ( $\text{Na}_2\text{SO}_4$ ). Filtration and concentration gave quantitative yield of a yellow oil that was used in the next step without further purification. The structure was verified by  $^1\text{H}$  and  $^{13}\text{C}$

NMR analysis.

iii) Synthesis of 4-[2-hydroxy-3-[(1-methyl-ethyl)amino]propoxy]phenylacetic acid hydrochloride

5

A solution of methyl 4-[2-hydroxy-3-[(1-methyl-ethyl)amino]propoxy]phenylacetate (563 mg, 2.00 mmol) in 6M hydrochloric acid (15 ml) was heated at 100 °C for 4h. The reaction mixture was concentrated (rotavapor) and the residue was taken up in water and lyophilised. <sup>1</sup>H and <sup>13</sup>C NMR spectra were in accordance with the structure and MALDI mass spectrometry gave a M+H at 268 as expected.

15

iv) Synthesis of N-Boc-4-[2-hydroxy-3-[(1-methyl-ethyl)amino]propoxy]phenylacetic acid

20

A solution of the 4-[2-hydroxy-3-[(1-methyl-ethyl)amino]propoxy]phenylacetic acid hydrochloride (2.0 mmol) in water (2 ml) was added to a solution of sodium bicarbonate (0.60 g, 7.2 mmol) in water/dioxane (2:1, 15 ml). A solution of di-tert-butyl dicarbonate (0.48 g, 2.2 mmol) in dioxane (5 ml) was added. Progress of the reaction was monitored by TLC analysis (silica, CHCl<sub>3</sub>/MeOH/AcOH 85:10:5), and portions of di-tert-butyl dicarbonate were added until conversion was complete. The reaction mixture was poured onto water saturated with potassium hydrogen sulphate and organic material was extracted into ethyl acetate. The organic phase was washed with water and brine, dried (Na<sub>2</sub>SO<sub>4</sub>) and filtered to give 0.6 g of crude material. The product was purified by chromatography (silica, CHCl<sub>3</sub>/MeOH/AcOH 85:10:5). The solution was concentrated and the residue was taken up in glacial acetic acid and lyophilised. Yield 415 mg (56%), white solid. The structure was confirmed by <sup>1</sup>H and <sup>13</sup>C NMR analysis.

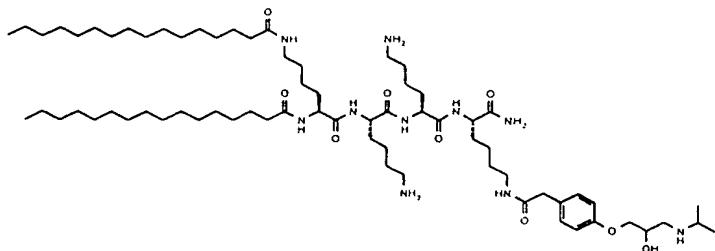
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b) Synthesis of a lipopeptide functionalised with atenolol

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10

The structure shown above was synthesised by the manual bubbler method starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale, using amino acids from Novabiochem, palmitic acid from Fluka 15 and the compound from a). Coupling was carried out using standard TBTU/HOBt/DIEA protocols.

Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT and 5% water for 2h. Crude 20 material was precipitated from ether and purified by preparative liquid chromatography (Vydac 218TP1022 column) using a gradient of 70 to 100% B over 60 min (A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 ml/min. After lyophilisation a yield of 25 38 mg of pure material was obtained (analytical HPLC: gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1 ml/min, column Vydac 218TP54, detection UV 214 nm, retention time 25 min). Further characterisation was carried out using 30 MALDI mass spectrometry (ACH matrix), giving M+H at 1258, expected 1257.

c) Preparation of gas-containing microbubbles of distearoyl-phosphatidyl-serine comprising a lipopeptide containing atenolol

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) and product from b) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then 5 heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no 10 detectable level of compound from b) in the final wash solution.

Incorporation of atenolol containing lipopeptide into the bubbles was confirmed by MALDI mass spectrometry as 15 follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI mass spectrometry (ACH-matrix), giving a M+H peak at 1259 corresponding to lipopeptide b).

20

d) In vitro study of gas-containing microbubbles of distearoyl-phosphatidyl-serine "doped" with a lipopeptide containing atenolol

25

The human endothelial cell line ECV 304, derived from a normal umbilical cord (ATCC CRL-1998) was cultured in 260 ml Nunc culture flasks in RPMI 1640 medium (Bio Whittaker) to which L-glutamine 200 mM, 30 penicillin/streptomycin (10.000 U/ml and 10.000 mcg/ml) and 10% fetal bovine serum (Hyclone Lot no. AFE 5183) were added.

The cells were subcultured with a split ratio of 1:5 to 1:7 when reaching confluence.

35

Cover-glasses, 22mm in diameter were sterilised and placed on the bottom of 12 well culture plates (Costar) before cells in 0,5 ml complete medium with serum was

added on top. When the cells reached confluence, the coverslips were placed in a custom made flow-chamber. The chamber has a groove carved into a built-in glass plate. The cover slip with cells was placed on this 5 plate with the cells facing the groove thus forming a flow channel.

Microbubbles from c) above were passed from a reservoir held at 37°C to the flow chamber and back to the reservoir through a peristaltic pump. The flow rate 10 could be adjusted to simulate any physiologically relevant shear rate. The flow chamber was placed under a microscope so that the interaction between the microspheres and the cells could be viewed directly. A camera mounted on the microscope was connected to a 15 colour video printer and a monitor.

A gradual accumulation of the microbubbles on the cells took place which depended on the flow rate. At increasing flow rates, the cells started to detach from the coverslip; the microbubbles were still bound to the 20 cells. Control bubbles not carrying the vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow conditions.

Example 13. Gas-filled microbubbles encapsulated with phosphatidylserine comprising a lipophilic derivative of atenolol with affinity for adrenergic receptors for diagnostic and/or therapeutic applications

a) Synthesis of N-hexadecyl-4-[2-hydroxy-3-[(1-methyl-30 ethyl)amino]propoxy]phenylacetamide

A solution of N-Boc-4-[2-hydroxy-3-[(1-methyl-ethoxy)amino]propoxy]phenylacetic acid (92 mg, 0.25 mmol) (prepared as in Example 12) and hexadecylamine (60 mg, 35 0.25 mmol) in DMF (5 ml) was cooled to 0 °C. HOBr (39 mg, 0.25 mmol) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (water soluble

carbodiimide) (48 mg, 0.25 mmol) were added. The reaction mixture was stirred at 0°C for 1 h and then at room temperature overnight. The reaction mixture was poured onto water (25 ml) containing sodium carbonate (2.5 g) and sodium chloride (4.0 g). Precipitated material was filtered off, washed with water, and taken up in chloroform. The chloroform phase was washed with 5% sodium carbonate and water and dried (Na<sub>2</sub>SO<sub>4</sub>). The solution was filtered and concentrated to give 150 mg of yellow-white crude material. The product was purified by column chromatography (silica, chloroform/methanol 95:5) to give 118 mg (80%) of white material. The structure was verified by <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz). The product was further characterised by MALDI mass spectrometry, giving a M+Na peak at 614 as expected.

vi) Synthesis of N-hexadecyl-4-[2-hydroxy-3-[(1-methyl-ethyl)amino]propoxy]phenylacetamide

To a solution of N'-Boc, N-hexadecyl-4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylacetamide (10 mg) in dichloromethane (9 ml) was added trifluoroacetic acid (1 ml). The reaction mixture was stirred for 2h at room temperature. TLC (silica, chloroform/methanol 95:5) showed complete conversion of starting material. Solvents were evaporated off and the residue was taken up in water/acetonitrile and lyophilised to give a quantitative yield of white solid material. The structure was verified by <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR analysis and further characterised by MALDI mass spectrometry, giving M+H at 492 and M+Na at 514 as expected.

b) Preparation of gas-filled microbubbles encapsulated with distearoyl-phosphatidylserine containing N-hexadecyl-4-[2-hydroxy-3-[(1-methylethyl)-aminolpropoxy]phenylacetamide

- 75 -

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) and N-hexadecyl-4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylacetamide (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming). The solution was filtered and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water.

Incorporation of compound from a) into the bubbles was confirmed by MALDI mass spectrometry as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI mass spectrometry, giving a M+H peak at 492 corresponding to N-hexadecyl-4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylacetamide

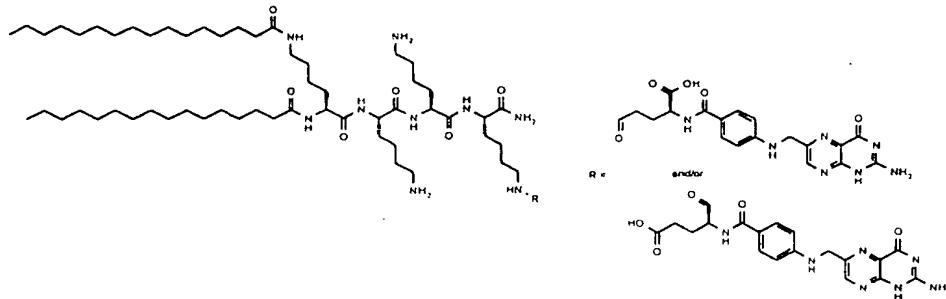
20

Example 14. Gas-filled microbubbles encapsulated with phosphatidyl-serine and a compound containing folic acid for diagnostic applications

25

a) Synthesis of a lipopeptide containing folic acid

30



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The structure shown above, with R, was synthesised by the manual bubbler method starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale, using amino acids from Novabiochem, palmitic acid from

- 76 -

Fluka and folic acid from Acros. Coupling was carried out using standard TBTU/HOBt/DIEA protocols.

Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried

5 out in TFA containing 5% EDT and 5% water for 2h. Crude material was precipitated from ether and analysed by MALDI mass spectrometry, giving a M+H peak corresponding to the structure at 1435, expected 1430. The material was further characterised by analytical HPLC (column

10 Vydac 218TP54, gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1.0 ml/min), giving a product peak with retention time 27 min detected at UV 368 nm.

15 b) Preparation of gas-containing microbubbles of distearoyl-phosphatidyl-serine comprising a lipopeptide containing folic acid

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0

20 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) and product from a) (0.5 mg) in a vial. Dilute ammonia (to pH 8) and DMSO (40  $\mu$ l) were added and the mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during

25 warming). The solution was filtered and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water.

30 Incorporation of structure from a) into the bubbles was confirmed by MALDI mass spectrometry as follows. Ca 50  $\mu$ l of microbubbles were transferred to a clean vial containing ca 100  $\mu$ l of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI mass

35 spectrometry (ACH matrix), giving a M+H peak at 1238 corresponding to structure from a).

c) In vitro study of gas-containing microbubbles of distearoyl-phosphatidyl-serine "doped" with a lipopeptide containing folic acid

5       The human endothelial cell line ECV 304 were grown as described in Example 12. Cover-glasses, 22mm in diameter, were sterilised and placed on the bottom of 12 well culture plates (Costar) before cells in 0.5 ml complete medium with serum was added on top.

10      When the cells reached confluence, the coverslips were placed in a custom-made flow-chamber. The chamber has a groove carved into a built-in glass plate. The cover slip with cells was placed on this plate with the cells facing the groove, thus forming a flow channel.

15      Microbubbles from b) above were passed from a reservoir held at 37°C to the flow chamber and back to the reservoir through a peristaltic pump. The flow rate could be adjusted to simulate any physiologically relevant shear rate. The flow chamber was placed under a microscope so that the interaction between the microspheres and the cells could be viewed directly. A camera mounted on the microscope was connected to a colour video printer and a monitor.

20      A gradual accumulation of the microbubbles on the cells took place which depended on the flow rate. At increasing flow rates, the cells started to detach from the coverslip; however, the microbubbles were still bound to the cells. Control bubbles not carrying the vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow conditions.

35      Example 15. Gas-filled microbubbles of distearoyl-phosphatidyl-serine containing a cholesteryl ester of chlorambucil for diagnostic and/or therapeutic

applicationsa) Synthesis of cholesteryl 4-[4-[bis(2-chloroethyl)aminophenyl]butanoate

5

DIC (170  $\mu$ l, 1.10 mmol) was added to a solution of chlorambucil (Sigma, 669 mg, 2.20 mmol) in dry dichloromethane (15 ml). The mixture was stirred at room temperature for 0.5 h and added to a solution of cholesterol (Aldrich, 387 mg, 1.00 mmol) and DMAP (122 mg, 1.00 mmol) in dichloromethane (10 ml). The reaction mixture was stirred overnight and then poured onto 5% sodium bicarbonate. The phases were separated and the organic phase was washed with brine and dried ( $MgSO_4$ ).  
10 The solution was filtered and concentrated and the product was purified by column chromatography (silica, chloroform) to give 560 mg (83%) yield of colourless oil. The product was characterised by MALDI mass spectrometry, giving  $M+H$  at 674 as expected. Further  
15 characterisation was carried out using  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) NMR analysis, giving spectra in accordance with the structure.

b) Preparation of gas-containing microbubbles of distearoyl-phosphatidyl-serine comprising a cholesteryl ester of chlorambucil for diagnostic and/or therapeutic applications

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) and product from a) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no

detectable level of compound from a) in the final wash solution.

5 Incorporation of chlorambucil cholesteryl ester into the bubbles was confirmed by MALDI mass spectrometry as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI mass spectrometry, giving a M+H peak at 668 corresponding to 10 structure from a).

15 Example 16. Gas-filled microbubbles encapsulated with phosphatidyl-serine comprising a lipopeptide containing atenolol and a cholesterol derivative of chlorambucil for diagnostic and therapeutic applications

20 This example is directed towards providing microbubbles comprising a non-peptidic vector for targeting, plus a therapeutic moiety.

25 A lipopeptide functionalised with atenolol was synthetized as in Example 12 and a cholesterol ester of chlorambucil was synthetized as in Example 15.

a) Preparation of microbubbles encapsulated with phosphatidyl-serine comprising a lipopeptide containing atenolol and a cholesteryl ester of chlorambucil

30 A solution of 1.4% propylene glycol/2.4% glycerol (1.0 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 5.0 mg), atenolol-functionalised lipopeptide (0.5 mg) and chlorambucil cholesteryl ester(0.5 mg) in a vial. The mixture was sonicated for 5 min and then warmed to 80 °C for 5 min (vial was shaken during warming). The solution was filtered and cooled. 35 The head space was flushed with perfluorobutane gas and

- 80 -

the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water.

Incorporation of atenolol-containing lipopeptide and  
5 chlorambucil cholesteryl into the bubbles was confirmed  
by MALDI mass spectrometry as follows. Ca 50 µl of  
microbubbles were transferred to a clean vial containing  
ca 100 µl of 90% methanol. The mixture was sonicated for  
30s and analysed by MALDI mass spectrometry using ACH-  
10 matrix.

b) In vitro study of gas-containing microbubbles  
encapsulated with phosphatidyl-serine comprising a  
lipopeptide containing atenolol and a cholesterol  
15 derivative of chlorambucil for diagnostic and  
therapeutic applications

The human endothelial cell line ECV 304 were grown as  
described in Example 12. Cover-glasses, 22mm in  
20 diameter, were sterilised and placed on the bottom of 12  
well culture plates (Costar) before cells in 0,5 ml  
complete medium with serum was added on top.

When the cells reached confluence, the coverslips  
were placed in a custom-made flow-chamber. The chamber  
25 has a groove carved into a built-in glass plate. The  
cover slip with cells was placed on this plate with the  
cells facing the groove thus forming a flow channel.

Microbubbles from a) above were passed from a  
reservoir held at 37°C to the flow chamber and back to  
30 the reservoir through a peristaltic pump. The flow rate  
could be adjusted to simulate any physiologically  
relevant shear rate. The flow chamber was placed under a  
microscope so that the interaction between the  
microspheres and the cells could be viewed directly. A  
35 camera mounted on the microscope was connected to a  
colour video printer and a monitor.

The microbubbles gradually accumulated on the

cells at a rate which depended on the flow rate. At increasing flow rates, the cells started to detach from the coverslip while the microbubbles were still bound to the cells. Control bubbles not carrying the vector did 5 not adhere to the endothelial cells and disappeared from the cells under minimal flow conditions.

10 Example 17. - Gas-filled microbubbles encapsulated with phosphatidyl-serine comprising a cholesterol derivative of atenolol for diagnostic and therapeutic applications

a) Synthesis of cholesteryl N-Boc- $\beta$ -alaninate

15 DIC (510  $\mu$ l) was added to a solution of Boc- $\beta$ -Ala-OH (1.25 g, 6.60 mmol) in dichloromethane (15 ml) under an inert atmosphere. The reaction mixture was stirred for 30 min and then transferred to a flask containing a solution of cholesterol (1.16 g, 3.00 mmol) and DMAP (367 mg, 3.00 mmol) in dichloromethane (15 ml). The 20 reaction mixture was stirred for 2 h and then poured onto an aqueous solution of potassium hydrogensulphate. Phases were separated and the aq phase was extracted with chloroform. Combined organic phases were washed with aq potassium hydrogensulphate and water and dried 25 ( $MgSO_4$ ). After filtration and evaporation the crude product was chromatographed (silica, chloroform/methanol 99:1) to give 1.63 g (97%) of white solid. The structure was confirmed by  $^1H$  NMR (500 MHz).

30 b) Synthesis of cholesteryl  $\beta$ -alaninate hydrochloride

A solution of the compound from a) (279 mg, 0.500 mmol) in 1M hydrochloric acid in 1,4-dioxan (5 ml) was stirred at room temperature for 4h. The reaction mixture was 35 concentrated to give a quantitative yield of cholesteryl  $\beta$ -alaninate hydrochloride. The structure was confirmed by  $^1H$  NMR (500 MHz) analysis and by MALDI mass

spectrometry, giving a M+Na peak at 482, expected 481.

c) Synthesis of cholesteryl N-Boc-

5   4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylacetyl β-alaninate.

To a solution of N-Boc-4-[2-hydroxy-3-[(1-methyl-  
10 ethyl)amino]propoxy]phenylacetic acid (55 mg, 0.15  
mmol) (synthetized as in Example 12) and cholesteryl β-  
alaninate hydrochloride (74 mg, 0.15 mmol) in DMF (5 ml)  
was added DIEA (26 ml, 0.15 mmol). HOEt (23 mg, 0.15  
mmol) and water soluble carbodiimide (WSC) (29 mg, 0.15  
mmol) was added. The reaction mixture was stirred at  
15 room temperature overnight and then poured onto water  
(25 ml) containing sodium carbonate (2.5 g) and sodium  
chloride (4.0 g). Precipitated material was extracted  
into chloroform. The organic phase was washed with water  
and dried ( $MgSO_4$ ). After filtration and concentration  
20 crude material (132 mg) was purified by column  
chromatography (silica, chloroform/methanol/acetic acid,  
95:4:1). Pooled fractions were concentrated, taken up in  
glacial acetic acid and lyophilised. Yield 83 mg (69%),  
yellow-white solid. Structure was confirmed by  $^1H$  NMR  
25 analysis

d) Synthesis of cholesteryl

4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenylacetyl β-alaninate trifluoroacetate

30 To a solution of N-Boc-4-[2-hydroxy-3-[(1-methyl-  
ethyl)amino]propoxy]phenylacetyl-β-alaninate (40 mg,  
0.05 mmol) in dry dichloromethane (4 ml) was  
trifluoroacetic acid (2 ml). The reaction mixture was  
35 stirred for 2 h and then concentrated. The product was  
lyophilised from a acetonitrile/water to give a  
quantitative yield of white-yellow material. The product

was characterised by MALDI mass spectrometry giving M+H at 708 as expected.

5           e) Preparation of gas-containing microbubbles  
encapsulated with phosphatidyl-serine containing a  
cholesterol derivative of atenolol for diagnostic and  
therapeutic applications

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0  
10 ml) was added to a mixture of Distearoyl-phosphatidyl-  
serine (Avanti, 4.5 mg) and product from d) (0.5 mg) in  
a vial. The mixture was sonicated for 5 min and then  
heated at 80 °C for 5 min (vial was shaken during  
warming) and cooled. Head space was flushed with  
15 perfluorobutane gas and the vial was shaken in a cap  
mixer for 45s followed by extensive washing with  
deionised water. MALDI mass spectrometry showed no  
detectable level of compound from b) in the final wash  
solution.

20           Incorporation of compound from d) into the bubbles was  
confirmed by MALDI mass spectrometry.

25           j) In vitro study of gas-containing microbubbles of  
Distearoyl-phosphatidyl-serine ~~doped~~ with a  
lipopeptide containing atenolol

The human endothelial cell line ECV 304 was cultured as  
in Example 12. Cover-glasses, 22mm in diameter were  
30 sterilised and placed on the bottom of 12-well culture  
plates (Costar) before cells in 0,5 ml complete medium  
with serum was added on top.

When the cells reached confluence the coverslips  
were placed in a custom-made flow-chamber. The chamber  
35 has a groove carved into a built-in glass plate. The  
cover slip with cells was placed on this plate with the  
cells facing the groove thus forming a flow channel.

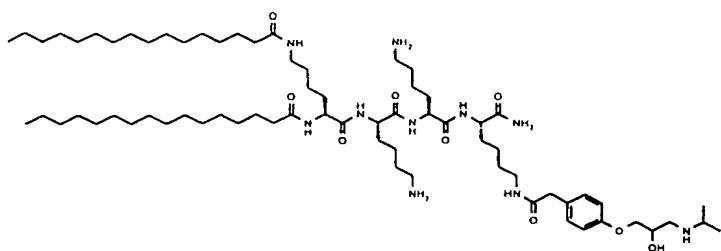
Ultrasound micro bubbles from d) above were passed from a reservoir held at 37°C to the flow chamber and back to the reservoir through a peristaltic pump. The flow rate could be adjusted to simulate any physiological relevant 5 shear rate. The flow chamber was placed under a microscope so that the interaction between the microspheres and the cells could be viewed directly. A camera mounted on the microscope was connected to a colour video printer and a monitor.

10 A gradual accumulation of the microbubbles on the cells took place which depended on the flow rate. At increasing flow rates, the cells started to detach from the coverslip; however, the microbubbles were still bound to the cells. Control bubbles not carrying the 15 vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow conditions.

Example 18. Gas-filled microbubbles of phosphatidyl-serine comprising a lipopeptide containing atenolol and a lipophilic derivative of captopril for diagnostic and therapeutic applications

a) Synthesis of a lipopeptide functionalised with 25 atenolol

30



The structure shown above was synthesised by the manual bubbler method as described in Example 12.

5       b) Synthesis of N-[S]-3-hexadecylthio-2-methylpropionyllproline

DIEA (188  $\mu$ l, 1.10 mmol) was added to a solution of 1-iodohexadecane (176 mg, 0.500 mmol), captopril (120 mg, 0.550 mmol) and DBU (165  $\mu$ l, 1.10 mmol) in tetrahydrofuran (5 ml). The mixture was heated at 70 °C for 2h and then concentrated. The residue was poured onto water saturated with potassium hydrogensulphate and organic material was extracted into chloroform. The organic phase was washed with water and dried ( $MgSO_4$ ).  
10      The product purified by chromatography (silica,  $CHCl_3/MeOH/AcOH$  85:10:5) and lyophilised to give 105 mg (48%) of white solid material. The structure was verified by  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) analysis and further characterised by MALDI mass spectrometry, giving  
15      M-H in negative mode at m/z 440 as expected.  
20

25       c) Preparation of gas-filled microbubbles encapsulated with phosphatidyl-serine comprising a lipopeptide containing atenolol and a lipophilic derivative of captopril for diagnostic and therapeutic applications

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 4.5 mg), product from a) (0.5 mg) and b) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no detectable level of compound from a) or b) in the final wash solution.  
30  
35

Incorporation of compound a) and b) into the bubbles was confirmed by MALDI-MS as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 5 30s and analysed by MALDI-MS using ACH-matrix.

d) In vitro study of gas-containing microbubbles of phosphatidyl-serine comprising a lipopeptide containing atenolol and a lipophilic derivative of captopril for diagnostic and therapeutic applications

The human endothelial cell line ECV 304 was cultured as in Example 12. Cover-glasses, 22mm in diameter were sterilised and placed on the bottom of 12-well culture 15 plates (Costar) before cells in 0,5 ml complete medium with serum was added on top.

When the cells reached confluence the coverslips were placed in a custom-made flow-chamber. The chamber has a groove carved into a built-in glass plate. The 20 cover slip with cells was placed on this plate with the cells facing the groove thus forming a flow channel.

Ultrasound micro bubbles from c) above were passed from a reservoir held at 37°C to the flow chamber and back to the reservoir through a peristaltic pump. The 25 flow rate could be adjusted to simulate any physiological relevant shear rate. The flow chamber was placed under a microscope so that the interaction between the microspheres and the cells could be viewed directly. A camera mounted on the microscope was connected to a colour video printer and a monitor.

A gradual accumulation of the microbubbles on the cells took place which depended on the flow rate. At increasing flow rates, the cells started to detach from the coverslip; however, the microbubbles were still 35 bound to the cells. Control bubbles not carrying the vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow

conditions.

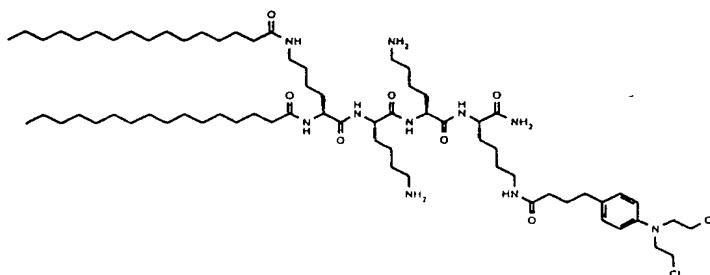
5

Example 19. - Gas-filled microbubbles encapsulated with Distearoyl-phosphatidyl-serine comprising a lipopeptide containing chlorambucil for diagnostic and therapeutic applications

10

a) Synthesis of a lipopeptide containing chlorambucil

15



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The structure shown above was synthesised by the manual bubbler method starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale. Standard amino acids were purchased from Novabiochem and palmitic acid from Fluka. Coupling was carried out using standard TBTU/HOBt/DIEA protocol. Chlorambucil (Sigma) was coupled through the side-chain of Lys as a symmetrical anhydride using DIC preactivation.

25

Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT, 5% water and 5% ethyl methyl sulphide for 2 h. An aliquot of 10 mg of the crude material was purified by preparative liquid chromatography (Vydac 218TP1022 column) using a gradient

of 70 to 100% B over 60 min (A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 ml/min. After lyophilisation a yield of 30 mg of pure material was obtained (analytical HPLC: gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile; flow rate 1 ml/min; column Vydac 218TP54; detection UV 214 nm; retention time 26.5 min). Further characterisation was carried out using MALDI mass spectrometry, giving M+H at 1295, expected 1294.

10

b) Preparation of gas-filled microbubbles comprising a lipopeptide containing chlorambucil for diagnostic and therapeutic applications

15

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) and product from a) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no detectable level of compound from a) in the final wash solution.

20

25

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Incorporation of chlorambucil containing lipopeptide into the bubbles was confirmed by MALDI-MS as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI-MS (ACH-matrix), giving a M+H peak at 1300, expected at 1294 and a M+Na peak at 1324, expected 1317.

35

c) In vitro study of gas-containing microbubbles of Distearoyl-phosphatidyl-serine ~~doped~~ with a lipopeptide containing chlorambucil for diagnostic and

therapeutic applications

The human endothelial cell line ECV 304 was cultured as in Example 12. Cover-glasses, 22mm in diameter were 5 sterilised and placed on the bottom of 12-well culture plates (Costar) before cells in 0,5 ml complete medium with serum was added on top.

When the cells reached confluence the coverslips were placed in a custom-made flow-chamber. The chamber 10 has a groove carved into a built-in glass plate. The cover slip with cells was placed on this plate with the cells facing the groove thus forming a flow channel.

Ultrasound micro bubbles from b) above were passed 15 from a reservoir held at 37°C to the flow chamber and back to the reservoir through a peristaltic pump. The flow rate could be adjusted to simulate any physiological relevant shear rate.

The flow chamber was placed under a microscope so that 20 the interaction between the microspheres and the cells could be viewed directly. A camera mounted on the microscope was connected to a colour video printer and a monitor.

A gradual accumulation of the microbubbles on 25 the cells took place which depended on the flow rate. At increasing flow rates, the cells started to detach from the coverslip; however, the microbubbles were still bound to the cells. Control bubbles not carrying the vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow 30 conditions.

Example 20. - Gas-filled microbubbles encapsulated with phosphatidylserine comprising biotinamide-PEG-β-Ala-Cholesterol and a cholesteryl ester of chlorambucil for diagnostic and therapeutic applications

a) Synthesis of cholesteryl β-alaninate

Cholesteryl  $\beta$ -alaninate hydrochloride was synthetized as in Example 17.

b) Biotin-PEG<sub>3400</sub>- $\beta$ -Ala-Cholesterol

5

To a solution of cholesteryl  $\beta$ -alaninate hydrochloride (15 mg, 0.03 mmol) in chloroform/wet methanol (2.6:1, 3 ml) was added triethylamine (42  $\mu$ l, 0.30 mmol). The mixture was stirred for 10 minutes at room temperature and a solution 10 of biotin-PEG3400-NHS (100 mg, 0.03 mmol) in 1,4-dioxane (1 ml) was added dropwise. After stirring at room temperature for 3 hours, the mixture was evapourated to dryness and the residue purified by flash chromatography to give white crystals, yield ; 102 mg (89%). The 15 structure was verified by MALDI-MS and by NMR analysis.

c) Synthesis of cholesteryl 4-[4-[bis(2-chloroethyl)aminophenyl]butanoate

20

DIC (170  $\mu$ l, 1.10 mmol) was added to a solution of chlorambucil (Sigma, 669 mg, 2.20 mmol) in dry dichloromethane (15 ml). The mixture was stirred at room temperature for 0.5 h and added to a solution of cholesterol (Aldrich, 387 mg, 1.00 mmol) and DMAP (122 mg, 1.00 mmol) in dichloromethane (10 ml). The reaction 25 mixture was stirred overnight and then poured onto 5% sodium bicarbonate. The phases were separated and the organic phase was washed with brine and dried ( $MgSO_4$ ). The solution was filtered and concentrated and the product was purified by column chromatography (silica, chloroform) to give 560 mg (83%) yield of colourless oil. The product was characterised by MALDI mass 30 spectrometry, giving M+H at 674 as expected. Further characterisation was carried out using  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) NMR analysis, giving spectra in accordance 35 with the structure.

d) Preparation of gas-filled microbubbles

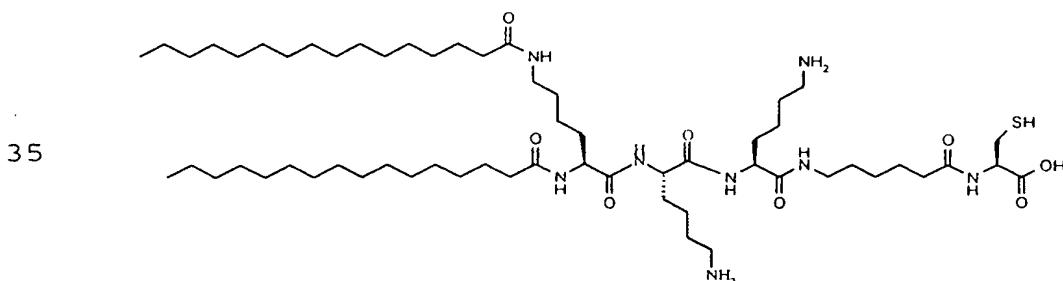
A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of DSPS (Avanti, 5 mg) and product from b) (0.5 mg) and c) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no detectable level of compound from b) and c) in the final wash solution.

Incorporation of compounds from b) and c) into the bubbles was confirmed by MALDI-MS as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI-MS (ACH-matrix).

Example 21: Preparation of thiol-functionalised gas-filled microbubbles for ultrasound imaging.

This example is directed to the preparation of microbubbles having a reactive group on the surface for non-specific attachment, principally utilising disulphide exchange reactions to effect binding to a multiplicity of cellular targets.

a) Synthesis of a thiol functionalised lipid molecule:  
Dipalmitoyl-Lys-Lys-Lys-6-amino-caproic acid-Cys



The lipid structure shown above was synthesised on a ABI 433A automatic peptide synthesiser starting with Fmoc-Cys(Trt)-Wang resin (Novabiochem) on a 0.25 mmol scale using 1 mmol amino acid cartridges. All amino acids and 5 palmitic acid were preactivated using HBTU coupling chemistry.

The simultaneous removal of peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT, and 5% H<sub>2</sub>O for 2 hours 10 giving a crude product yield of 250 mg. Purification by preparative HPLC (Vydac 218TP1022 column) of a 40 mg aliquot of crude material was carried out using a gradient of 90 to 100 % B over 50 min (A= 0.1 % TFA/water and B = MeOH) at a flow rate of 9 mL/min. 15 After lyophilization 24 mg of pure material was obtained (Analytical HPLC; Gradient, 70-100%B where B= 0.1% TFA/acetonitrile, A= 0.01% TFA/water: column - vydac 218TP54: Detection - UV 214 nm-product retention time = 23 min). Further product characterization was carried 20 out using MALDI mass spectrometry; expected, M+H at 1096, found, at 1099.

b) Preparation of thiol-functionalised gas-filled microbubbles

25 DSPS (Avanti, 5.0 mg) and the thiol containing lipid structure from example 15 a)(1.0 mg) were weighed into a clean vial and 0.8 mL of a solution containing 1.4% propylene glycol/ 2.4% glycerol in water added. The 30 mixture was warmed to 80°C for 5 minutes (vials shaken during warming) and filtered while still hot through a 40 micron filter. The samples were cooled to room temperature and the head space flushed with perfluorobutane gas. The vials were shaken in a cap 35 mixer for 45 s and the microbubbles placed on roller table overnight. Bubbles were washed several times with deionised water and analysed for thiol group

incorporation using Ellman's reagent.

5           Example 22: Preparation of microbubbles coated with  
poly-L-lysine complexed to fluorescein labeled DNA  
fragments from plasmid pBR322

This example is directed to the preparation of  
microbubbles for gene therapy/anti-sense applications.  
It is envisaged that specific targeting may be achieved  
10 by further doping of microbubble membranes with vector  
modified lipid structures as described in example 1.

15           a) Preparation of gas-containing microbubbles  
encapsulated with phosphatidyl-serine

Distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) was  
weighed into a clean vial. 1.0 mL of a solution of 1.4%  
propylene glycol/ 2.4% glycerol was added and the  
mixture sonicated for 2 min, then warmed to 80°C for 5  
20 minutes. Immediately following warming, the solution  
was filtered through a 4 micron filter. The sample was  
cooled to room temperature and the head space flushed  
with perfluorobutane gas. The vial was shaken in a cap  
mixer for 45 s. Bubbles were then washed once with  
25 deionised water and the infranatant discarded. The  
microbubbles were then resuspended in 0.5 mL water.

30           b) Preparation of poly-L-lysine/DNA complex and loading  
of phosphatidyl-serine microbubbles

To 1 mg of poly-L-lysine (70-150 kD) in a clean vial was  
added 0.1 mL of a fluorescein labeled digest of plasmid  
pBR322 (Biorad) dissolved in TE buffer (10 mM tris-HCl,  
pH 8). The solution was made up to a total of 0.6 mL  
35 by addition of water and the pH adjusted to 8.  
Complexation was allowed to proceed for 1 h then 0.05 mL  
of the polylysine-DNA solution was added to the

microbubble suspension from a) above. After 1 h microscopy was used to show that the bubbles were fluorescent confirming the presence of DNA.

5       Example 23 - Gas-filled microbubbles of phosphatidyl-serine comprising a lipopeptide containing atenolol for cell targeting and a lipophilic thiol ester of captopril for therapeutic use.

10

a) Synthesis of cholic acid thiol ester of captopril

A mixture of 5- $\beta$ -cholic acid (Sigma, 361 mg, 1.00 mmol) and DIC (77 $\mu$ l, 0.50 mmol) in dichloromethane (5 ml) was stirred for 10 min and then added to a solution of captopril (Sigma, 130 mg, 0.600 mmol) and DBU (180  $\mu$ l, 1.20 mmol) in dichloromethane (10 ml). The reaction mixture was stirred overnight and then poured onto dilute hydrochloric acid. Chloroform (30 ml) was added. The phases were separated and the organic phase was washed with water and brine and dried ( $MgSO_4$ ). After filtration and concentration the crude material was chromatographed (silica, chloroform/methanol/acetic acid 95:4:1). The product was lyophilised from a acetonitrile/water/ethanol mixture. Yield 137 mg (49%) of off-white solid. The structure was verified by  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) NMR spectroscopy. Further characterisation was carried out using MALDI mass spectrometry, giving a M+Na peak in positive mode at m/z 584.

b) Preparation of gas-filled microbubbles of phosphatidyl-serine comprising a lipopeptide containing atenolol for cell targeting and a lipophilic thiol ester of captopril for therapeutic use.

A solution of 1.4% propylene glycol / 2.4% glycerol (1.0 ml) was added to a mixture of Distearoyl-phosphatidyl-serine (Avanti, 5.0 mg) and product from a) (0.5 mg) and lipopeptide containing atenolol from Example 12 (0.5 mg) 5 in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no detectable level of compound from b) and c) in the final wash solution.

10 Incorporation of compounds from a) and lipopeptide containing atenolol into the bubbles was confirmed by 15 MALDI-MS.

e) In vitro study of gas-containing microbubbles of phosphatidyl-serine 'doped' with a lipopeptide containing atenolol

20 The human endothelial cell line ECV 304 were grown as described in Example 12. Cover-glasses, 22mm in diameter, were sterilised and placed on the bottom of 12 well culture plates (Costar) before cells in 0.5 ml 25 complete medium with serum was added on top.

When the cells reached confluence, the coverslips were placed in a custom-made flow-chamber. The chamber has a groove carved into a built-in glass plate. The cover slip with cells was placed on this plate with the 30 cells facing the groove, thus forming a flow channel.

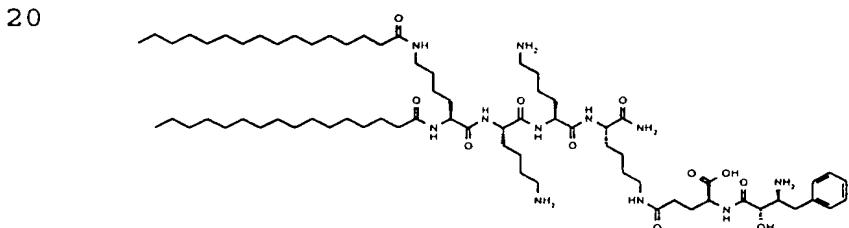
35 Microbubbles from b) above were passed from a reservoir held at 37°C to the flow chamber and back to the reservoir through a peristaltic pump. The flow rate could be adjusted to simulate any physiologically relevant shear rate. The flow chamber was placed under a microscope so that the interaction between the microspheres and the cells could be viewed directly. A

camera mounted on the microscope was connected to a colour video printer and a monitor.

A gradual accumulation of the microbubbles on the cells took place which depended on the flow rate. At 5 increasing flow rates, the cells started to detach from the coverslip; however, the microbubbles were still bound to the cells. Control bubbles not carrying the vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow 10 conditions.

Example 24 - Gas-filled microbubbles encapsulated with phosphatidyl-serine comprising a lipopeptide containing a derivative of bestatin for diagnostic and therapeutic applications

a) Synthesis of a lipopeptide containing a derivative of bestatin



The structure shown above was synthesised by the manual bubbler method starting with Fmoc protected Rink Amide MBHA resin (Novabiochem) on a 0.125 mmol scale, using amino acids from Novabiochem and palmitic acid from Fluka. Coupling was carried out using standard TBTU/HOBt/DIEA protocols.

Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT and 5% water for 2h. Crude material was precipitated from ether and purified by preparative liquid chromatography (Vydac 218TP1022 column) using a gradient of 70 to 100% B over 60 min (A

= 0.1% TFA/water and B = 0.1% TFA/acetonitrile) at a flow rate of 10 ml/min. After lyophilisation a yield of 12 mg of pure material was obtained (analytical HPLC: gradient 70-100% B over 20 min, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1 ml/min, column Vydac 218TP54, detection UV 214 nm, retention time 25 min). Further characterisation was carried out using MALDI mass spectrometry (ACH matrix), giving M+H at 1315, expected 1314.

10

b) Preparation of gas-filled microbubbles comprising a lipopeptide containing a derivative of bestatin for diagnostic and therapeutic applications

15

A solution of 1.4% propylene glycol/2.4% glycerol (1.0 ml) was added to a mixture of Distearoyl-phosphatidyl-serine (Avanti, 4.5 mg) and product from a) (0.5 mg) in a vial. The mixture was sonicated for 5 min and then heated at 80 °C for 5 min (vial was shaken during warming) and cooled. Head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45s followed by extensive washing with deionised water. MALDI mass spectrometry showed no detectable level of compound from a) in the final wash solution.

20

25

Incorporation of atenolol containing lipopeptide into the bubbles was confirmed by MALDI-MS as follows. Ca 50 µl of microbubbles were transferred to a clean vial containing ca 100 µl of 90% methanol. The mixture was sonicated for 30s and analysed by MALDI-MS (ACH-matrix), giving a M+H peak at 1320, expected at 1314, corresponding to lipopeptide from a).

30

35

c) In vitro study of gas-containing microbubbles of Distearoyl-phosphatidyl-serine ~~doped~~ with a lipopeptide containing a derivative of bestatin for

diagnostic and therapeutic applications

5       The human endothelial cell line ECV 304 were grown as described in Example 12. Cover-glasses, 22mm in diameter, were sterilised and placed on the bottom of 12 well culture plates (Costar) before cells in 0.5 ml complete medium with serum was added on top.

10      When the cells reached confluence, the coverslips were placed in a custom-made flow-chamber. The chamber has a groove carved into a built-in glass plate. The cover slip with cells was placed on this plate with the cells facing the groove, thus forming a flow channel.

15      Microbubbles from b) above were passed from a reservoir held at 37°C to the flow chamber and back to the reservoir through a peristaltic pump. The flow rate could be adjusted to simulate any physiologically relevant shear rate. The flow chamber was placed under a microscope so that the interaction between the microspheres and the cells could be viewed directly. A 20     camera mounted on the microscope was connected to a colour video printer and a monitor.

25      A gradual accumulation of the microbubbles on the cells took place which depended on the flow rate. At increasing flow rates, the cells started to detach from the coverslip; however, the microbubbles were still bound to the cells. Control bubbles not carrying the vector did not adhere to the endothelial cells and disappeared from the cells under minimal flow conditions.

30

Example 25 - Gas-filled microbubbles of distearoyl-phosphatidylserine comprising a lipopeptide containing a vector with affinity for endothelin receptors for targeted ultrasound imaging

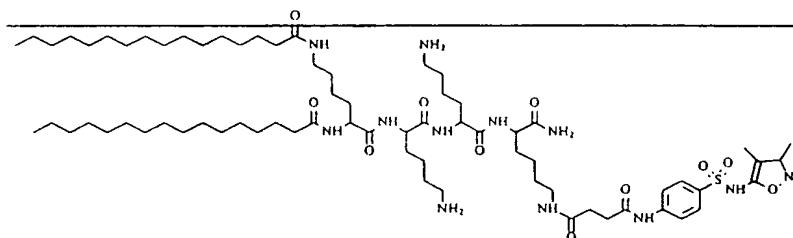
35

a) Synthesis of 4'-(3,4-dimethyl-5-isoxazolyl)-sulfamoyllsuccinanilic acid

To a solution of sulfisoxazole (267 mg, 1.00 mmol) in DMF (10 ml) was added succinic anhydride (1.00 g, 10.0 mmol) and 4-dimethylaminopyridine (122 mg, 1.00 mmol). The reaction mixture was stirred at 80°C for 2 hours and then concentrated. The residue was taken up in 5% aqueous sodium bicarbonate solution and extracted with ethyl acetate. The aqueous solution was acidified with dilute hydrochloric acid and organic material was extracted into ethyl acetate. The organic phase was washed with dilute hydrochloric acid, water and brine, treated with active charcoal and dried ( $\text{MgSO}_4$ ). The solution was filtered and concentrated to give 280 mg (76%) of white solid. The structure was verified by  $^1\text{H}$  (300 MHz) and  $^{13}\text{C}$  (75 MHz) NMR spectroscopy. Further characterisation was carried out using MALDI mass spectrometry (ACH matrix), giving a M+Na peak at m/z 390 and a M+K peak at m/z 406 as expected.

b) Synthesis of a lipopeptide functionalised with sulfisoxazole

25



30

35

The structure shown above was synthesised on a manual nitrogen bubbler apparatus starting with Fmoc-protected Rink Amide BMHA resin on a 0.125 mmol scale, using appropriate amino acids, palmitic acid and the compound from (a). Coupling was carried out using standard TBTU/HOBt/DIEA protocols. Simultaneous removal of the peptide from the resin and deprotection of side-chain protecting groups was carried out in TFA containing 5% EDT and 5% water for 2 hours. Crude material was

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precipitated from ether. The product was analysed by analytical HPLC, gradient 70-100% B over 20 minutes, A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile, flow rate 1 ml/minute, detection UV 214 nm, retention time 27 minutes). Further characterisation was carried out using MALDI mass spectrometry, giving a M+H at m/z 1359, expected 1356.

5           c) Preparation of gas-filled microbubbles comprising  
10          the compound from (b)

A solution of 1.4% propylene glycol/2.4% glycerol (1.0 ml) was added to a mixture of DSPS (4.5 mg) and product from (b) (0.5 mg) in a vial. The mixture was sonicated 15 for 5 minutes and then heated at 80°C for 5 minutes (vial was shaken during warming) and cooled. The head space was flushed with perfluorobutane gas and the vial was shaken in a cap mixer for 45 seconds followed by extensive washing with deionised water. MALDI mass 20 spectrometry showed no detectable level of compound from (b) in the final wash solution. Incorporation of isoxazole-containing lipopeptide into the microbubbles was confirmed by MALDI-MS as follows: ca. 50 µl of 25 microbubbles were transferred to a clean vial containing ca. 100 µl of 90% methanol. The mixture was sonicated for 30 seconds and analysed by MALDI-MS (ACH-matrix), giving a m+H peak at m/z 1359 corresponding to lipopeptide (b).

Claims

1. A targetable diagnostic and/or therapeutically active agent comprising a suspension in an aqueous carrier liquid of a reporter comprising gas-containing or gas-generating material, said reporter being conjugated to one or more non-proteinaceous, non-peptide and non-polysaccharide vectors.
- 10 2. An agent as claimed in claim 1 wherein the gas comprises air, nitrogen, oxygen, carbon dioxide, hydrogen, an inert gas, a sulphur fluoride, selenium hexafluoride, a low molecular weight hydrocarbon, a ketone, an ester, a halogenated low molecular weight hydrocarbon or a mixture of any of the foregoing.
- 15 3. An agent as claimed in claim 2 wherein the gas comprises a perfluorinated ketone, perfluorinated ether or perfluorocarbon.
- 20 4. An agent as claimed in claim 2 wherein the gas comprises sulphur hexafluoride or a perfluoropropane, perfluorobutane or perfluoropentane.
- 25 5. An agent as claimed in any of the preceding claims comprising gas microbubbles stabilised by a coalescence-resistant surface membrane, a filmogenic protein, a polymer material, a non-polymeric and non-polymerisable wall-forming material or a surfactant.
- 30 6. An agent as claimed in claim 5 wherein said surfactant comprises at least one phospholipid.
- 35 7. An agent as claimed in claim 6 wherein at least 75% of the said surfactant material comprises phospholipid molecules individually bearing net overall charge.

8. An agent as claimed in claim 7 wherein at least 75% of the film-forming surfactant material comprises one or more phospholipids selected from 5 phosphatidylserines, phosphatidylglycerols, phosphatidylinositol, phosphatidic acids and cardiolipins.

9. An agent as claimed in claim 8 wherein at least 10 80% of said phospholipids comprise phosphatidylserines.

10. An agent as claimed in any of the preceding claims wherein said vector or vectors are non-polymeric and synthetic or semi-synthetic. 15

11. An agent as claimed in claim 10 wherein said vector or vectors are exogenous to the human body.

12. An agent as claimed in any of claims 1 to 9 20 wherein said vector or vectors are selected from oligonucleotides and polynucleotides.

13. An agent as claimed in any of claims 1 to 9 25 wherein the vector or vectors are selected from non-peptide agonists/antagonists and binders of receptors for cell adhesion molecules, cytokines, growth factors and peptide hormones; oligonucleotides and modified oligonucleotides; DNA-binding drugs; protease substrates/inhibitors; non-peptide molecules generated from combinatorial libraries; and small bioactive 30 molecules.

14. An agent as claimed in any of the preceding claims 35 wherein the vector or vectors have affinity for targets at a level such that the agent interacts with but does not fixedly bind to said targets.

15. An agent as claimed in claim 14 wherein the vector or vectors are selected from ligands for cell adhesion proteins and cell adhesion proteins which have corresponding ligands on endothelial cell surfaces or  
5 from non-peptide ligands for cell adhesion proteins.

16. An agent as claimed in any of the preceding claims wherein the vector or vectors are sited such that they are not readily exposed to the target.  
10

17. An agent as claimed in any of the preceding claims wherein the vector is covalently or non-covalently coupled or linked to the reporter.

15 18. An agent as claimed in any one of claims 1 to 16 wherein the vector is coupled or linked to the reporter by means of electrostatic charge interaction.

19. An agent as claimed in any one of claims 1 to 16  
20 wherein the vector is coupled or linked to the reporter by means of avidin-biotin and/or streptavidin-biotin interactions.

20. An agent as claimed in any of the preceding claims which further contains moieties which are radioactive or are effective as X-ray contrast agents, light imaging probes or spin labels.  
25

21. An agent as claimed in any of the preceding claims further comprising a therapeutic compound.  
30

22. An agent as claimed in claim 21 wherein said therapeutic compound is an antineoplastic agent, blood product, biological response modifier, antifungal agent,  
35 hormone or hormone analogue, vitamin, enzyme, antiallergic agent, tissue factor inhibitor, platelet inhibitor, coagulation protein target inhibitor, fibrin

formation inhibitor, fibrinolysis promoter, antiangiogenic, circulatory drug, metabolic potentiator, antitubercular, antiviral, vasodilator, antibiotic, antiinflammatory, antiprotozoan, antirheumatic, 5 narcotic, opiate, cardiac glycoside, neuromuscular blocker, sedative, local anaesthetic, general anaesthetic or genetic material.

23. An agent as claimed in claim 21 or claim 22  
10 wherein said therapeutic compound is covalently coupled or linked to the reporter through disulphide groups.

24. An agent as claimed in claim 21 or claim 22  
wherein a lipophilic or lipophilically-derivatised  
15 therapeutic compound is linked to the reporter through hydrophobic interactions.

25. A combined formulation comprising:  
i) a first administrable composition  
20 comprising a pre-targeting vector having affinity for a selected target; and  
ii) a second administrable composition comprising an agent as claimed in any of the preceding claims, said agent comprising a vector having affinity 25 for said pre-targeting vector.

26. A combined formulation as claimed in claim 25  
wherein said pre-targeting vector comprises a derivatised mono- or oligosaccharide.  
30

27. A combined formulation comprising:  
i) a first administrable composition comprising an agent as claimed in any of claims 1 to 24,  
35 and  
ii) a second administrable composition comprising a substance capable of displacing or releasing said agent from its target.

28. A combined formulation comprising:

i) a first administrable composition comprising an agent as claimed in claim 23, and  
ii) a second administrable composition comprising a reducing agent capable of reductively cleaving the disulphide groups coupling or linking the therapeutic compound and reporter in the agent of said first administrable composition.

10 29. A process for the preparation of a targetable diagnostic and/or therapeutically active agent as defined in claim 1 which comprises coupling or linking at least one non-proteinaceous, non-peptide and non-polysaccharide vector to a reporter comprising gas-containing or gas-generating material.

15 30. A process as claimed in claim 29 wherein a therapeutic compound is also combined with the reporter.

20 31. Use of an agent as claimed in any of claims 1 to 24 as a targetable ultrasound contrast agent.

25 32. A method of generating enhanced images of a human or non-human animal body which comprises administering to said body an agent as claimed in any of claims 1 to 24 and generating an ultrasound, magnetic resonance, X-ray, radiographic or light image of at least a part of said body.

30 33. A method as claimed in claim 32 which comprises the steps:

i) administering to said body a pre-targeting vector having affinity for a selected target; and thereafter

35 ii) administering an agent as claimed in any of claims 1 to 24, said agent comprising a vector having affinity for said pre-targeting vector.

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34. A method as claimed in claim 33 wherein said pre-targeting vector comprises a derivatised mono- or oligosaccharide.

5 35. A method as claimed in claim 32 which comprises the steps:

i) administering to said body an agent as claimed in any of claims 1 to 24; and thereafter

10 ii) administering a substance capable of displacing or releasing said agent from its target.

36. A method as claimed in any of claims 32 to 35 wherein said agent further comprises a therapeutic compound.

15 37. A method as claimed in claim 36 wherein said therapeutic compound is covalently coupled or linked to the reporter through disulphide groups, and a composition comprising a reducing agent capable of 20 reductively cleaving said disulphide groups is subsequently administered.

25 38. A method for *in vitro* investigation of targeting by an agent as defined in any of claims 1 to 24 wherein cells expressing a target are fixedly positioned in a flow chamber, a suspension of said agent in a carrier liquid is passed through said chamber, and binding of said agent to said cells is examined.

30 39. A method as claimed in claim 38 wherein the flow rate of carrier liquid is controlled to simulate shear rates encountered *in vivo*.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 49/00, 47/48, 51/12</b>		(11) International Publication Number: <b>WO 98/18495</b>																					
		<b>A3</b>																					
		(43) International Publication Date: 7 May 1998 (07.05.98)																					
<p>(21) International Application Number: <b>PCT/GB97/02955</b></p> <p>(22) International Filing Date: 28 October 1997 (28.10.97)</p> <p>(30) Priority Data:</p> <table> <tr><td>9622365.6</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9622367.2</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9622366.4</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9700699.3</td><td>15 January 1997 (15.01.97)</td><td>GB</td></tr> <tr><td>9708265.5</td><td>24 April 1997 (24.04.97)</td><td>GB</td></tr> <tr><td>9711842.6</td><td>6 June 1997 (06.06.97)</td><td>GB</td></tr> <tr><td>9711845.9</td><td>6 June 1997 (06.06.97)</td><td>GB</td></tr> </table> <p>(71) Applicant (for GB only): MARSDEN, John, Christopher [GB/GB]; Frank B. Dehn &amp; Co., 179 Queen Victoria Street, London EC4V 4EL (GB).</p> <p>(71) Applicant (for all designated States except US): NYCOMED IMAGING AS [NO/NO]; Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): KLAVENESS, Jo [NO/NO]; Midtåsen 5, N-1166 Oslo (NO). RONGVED, Pål [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). HØGSET,</p>			9622365.6	28 October 1996 (28.10.96)	GB	9622367.2	28 October 1996 (28.10.96)	GB	9622366.4	28 October 1996 (28.10.96)	GB	9700699.3	15 January 1997 (15.01.97)	GB	9708265.5	24 April 1997 (24.04.97)	GB	9711842.6	6 June 1997 (06.06.97)	GB	9711845.9	6 June 1997 (06.06.97)	GB
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(54) Title: IMPROVEMENTS IN OR RELATING TO DIAGNOSTIC/THERAPEUTIC AGENTS

## (57) Abstract

Targetable diagnostic and/or therapeutically active agents, e.g. ultrasound contrast agents, comprising a suspension in an aqueous carrier liquid of a reporter comprising gas-containing or gas-generating material, said reporter being conjugated to one or more non-proteinaceous, non-peptide and non-polysaccharide vectors.

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/02955

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 A61K49/00 A61K47/48 A61K51/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 41647 A (BARNES JEWISH HOSPITAL D B A) 27 December 1996 see page 12, line 28 - page 13, line 10; claims 1,4,5,10; examples see page 14, line 18 - line 34; claims ---	1-39
E	WO 98 00172 A (UNIV NEBRASKA) 8 January 1998 see claims 1,15 ---	1-39
X	WO 91 15244 A (SINTETICA SA) 17 October 1991 see claims 1,4,7,25-27 ---	1-39

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the international search  6 May 1998	Date of mailing of the international search report  11.06.98
Name and mailing address of the ISA  European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer  Berte, M

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02955

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>DATABASE BIOSIS          BIOSCIENCES INFORMATION SERVICE,          PHILADELPHIA, PA, US          AN=98:17719,</p> <p>VILLANUEVA F.S. ET AL.: "MICROBUBBLES          TARGETED TO INTERCELLULAR ADHESION          MOLECULE-1 BIND TO ACTIVATED CORONARY          ARTERY ENDOTHELIUM FUNCTION USING          MYOCARDIAL CONTRAST ECHOCARDIOGRAPHY."          XP002063693          see abstract          &amp;          CIRCULATION,          vol. 96, no. 8SUPPL., 1997,          pages I457-I458,</p> <p>---</p>	1-37
P,X	<p>WO 97 33474 A (UNIV NEBRASKA) 18 September          1997          see claims 1,2,10,14,15,21,26,27</p> <p>---</p>	1-39
P,X	<p>DATABASE MEDLINE          US NATIONAL LIBRARY OF MEDICINE (NLM),          BETHESDA, MD, US          AN=97384421,          XP002064110          see abstract          &amp; KLIBANOV A.L. ET AL.: "TARGETING OF          ULTRASOUND CONTRAST MATERIAL. AN IN VITRO          FEASIBILITY STUDY."          ACTA RADILOGICA. SUPPLEMENTUM,          vol. 412, 1997,          pages 113-120,</p> <p>-----</p>	1-39

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Information on patent family members

International Application No

PCT/GB 97/02955

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9733474 A	18-09-97	AU 2207197 A	01-10-97





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 49/00, 47/48, 51/12</b>		<b>A3</b>	(11) International Publication Number: <b>WO 98/18495</b>																																	
			(43) International Publication Date: <b>7 May 1998 (07.05.98)</b>																																	
<p>(21) International Application Number: <b>PCT/GB97/02955</b></p> <p>(22) International Filing Date: <b>28 October 1997 (28.10.97)</b></p> <p>(30) Priority Data:</p> <table> <tr><td>9622365.6</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9622367.2</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9622366.4</td><td>28 October 1996 (28.10.96)</td><td>GB</td></tr> <tr><td>9700699.3</td><td>15 January 1997 (15.01.97)</td><td>GB</td></tr> <tr><td>9708265.5</td><td>24 April 1997 (24.04.97)</td><td>GB</td></tr> <tr><td>9711842.6</td><td>6 June 1997 (06.06.97)</td><td>GB</td></tr> <tr><td>9711845.9</td><td>6 June 1997 (06.06.97)</td><td>GB</td></tr> </table> <p>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications</p> <table> <tr><td>US</td><td>60/049,267 (CIP)</td></tr> <tr><td>Filed on</td><td>6 June 1997 (06.06.97)</td></tr> <tr><td>US</td><td>60/049,265 (CIP)</td></tr> <tr><td>Filed on</td><td>6 June 1997 (06.06.97)</td></tr> <tr><td>US</td><td>60/049,264 (CIP)</td></tr> <tr><td>Filed on</td><td>6 June 1997 (06.06.97)</td></tr> </table> <p>(71) Applicant (<i>for GB only</i>): MARSDEN, John, Christopher [GB/GB]; Frank B. Dehn &amp; Co., 179 Queen Victoria Street, London EC4V 4EL (GB).</p> <p>(71) Applicant (<i>for all designated States except US</i>): NYCOMED IMAGING AS [NO/NO]; Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): KLAVENESS, Jo [NO/NO]; Midtåsen 5, N-1166 Oslo (NO). RONGVED, Pål [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). HØGSET, Anders [NO/NO]; Treskevn 32A, N-0681 Oslo (NO). TOLLESHAUG, Helge [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). GODAL, Aslak [NO/NO]; Nedre Silkestrå 16, N-0365 Oslo (NO). LØVHAUG, Dagfinn [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). SOLBAKKEN, Magne [NO/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO). CUTHERBERTSON, Alan [GB/NO]; Nycomed Imaging AS, Nycoveien 2, P.O. Box 4220 Torshov, N-0401 Oslo (NO).</p> <p>(74) Agent: MARSDEN, John, Christopher; Frank B. Dehn &amp; Co., 179 Queen Victoria Street, London EC4V 4EL (GB).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b>  <i>With international search report.</i>  <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: <b>30 July 1998 (30.07.98)</b></p>				9622365.6	28 October 1996 (28.10.96)	GB	9622367.2	28 October 1996 (28.10.96)	GB	9622366.4	28 October 1996 (28.10.96)	GB	9700699.3	15 January 1997 (15.01.97)	GB	9708265.5	24 April 1997 (24.04.97)	GB	9711842.6	6 June 1997 (06.06.97)	GB	9711845.9	6 June 1997 (06.06.97)	GB	US	60/049,267 (CIP)	Filed on	6 June 1997 (06.06.97)	US	60/049,265 (CIP)	Filed on	6 June 1997 (06.06.97)	US	60/049,264 (CIP)	Filed on	6 June 1997 (06.06.97)
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